STRUCTURAL AND FUNCTIONAL CHARACTERIZATION OF THE PHOSPHOPROTEIN SUBUNIT OF THE SENDAI VIRUS RNA-DEPENDENT RNA POLYMERASE

By

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KEY TO SYMBOLS

aa	amino acid
ATP	adenosine 5'triphosphate
bp	base pair
C-	carboxy
°C	degrees Celsius
cfu	colony forming units
Ci	Curie
cm	centimeter
cpm	counts per minute
CTP	cytidine 5'-triphosphate
DI	defective interfering particle
DNA	deoxyribonucleic acid
ds	double stranded
GTP	guanosine 5'-triphosphate
h	hour
I.U.	International unit
kb	kilobase
kDa	kilodalton
le-	antigenomic leader ribonucleic acid
le+	genomic leader ribonucleic acid
MEM	minimal essential media

µCi	microcurie
µg	microgram
µl	microliter
µm	micrometer
µM	micromolar
mCi	millicurie
mg	milligram
ml	milliliter
mm	millimeter
mM	millimolar
min	minute
m.o.i.	multiplicity of infection
mRNA	messenger ribonucleic acid
(-)	negative sense
ng	nanogram
nm	nanometer
nt	nucleotide
OD	optical density
^{32}P	phosphorus 32
pfu	plaque forming units
(+)	positive sense
RNA	ribonucleic acid
rpm	revolutions per minute
rt	room temperature
^{35}S	sulfur 35
s	second

ss	single stranded
TTP	thymidine 5′-triphosphate
tRNA	transfer ribonucleic acid
U	units
UV	ultraviolet
UTP	uridine 5′-triphosphate
v/v	volume per volume
w/v	weight per volume
w/w	weight per weight

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The phosphorylated Sendai virus P protein is an essential component of the viral RNA polymerase (P-L complex) required for RNA synthesis. To identify amino acids important for P-L binding, site-directed mutagenesis of the P gene changed 20 charged amino acids singly or in groups within the L binding domain from amino acids 426-473. Of 10 mutants, each was wildtype for P-L and P-P protein interactions, yet several showed a severe inhibition of *in vitro* transcription. To determine if binding was instead hydrophobic in nature, 3 conserved hydrophobic residues were also mutated. Each also retained the ability to bind L, yet two gave a significant decrease in transcription. Wildtype biological activity could be restored for two inactive mutants by the addition of wild

type P protein, while the activity of two others could not be rescued. Gradient sedimentation analyses showed that rescue was not due to exchange of the wild type and mutant P proteins within the P-L complex. Mutants which gave a defective RNA synthesis phenotype and could not be rescued should help to establish an as yet unknown role for P within the polymerase complex, while the mutants which could be rescued will define a supplemental role for the P protein independent of polymerase function. Mutational analysis was also undertaken to determine the role of P phosphorylation. A major phosphorylation site, serine 249, was changed to alanine (S249A) which eliminated phosphorylation. This mutation retained wildtype protein:protein interaction and RNA synthesis activities. Thus, phosphorylation of the P protein at S249 is unnecessary for RNA synthesis *in vitro*.

CHAPTER 1
INTRODUCTION

A virus, consisting of genetic material enclosed in a protective coating, is one of the simplest entities able to reproduce. Viruses have no metabolic systems, they have no intrinsic mobility, they cannot respond to stimuli, and they do not grow in the usual sense. The ability to maintain genetic continuity, with the possibility for mutation, is the only basis for considering viruses to be alive.

Furthermore, the mechanism by which viruses reproduce is unique in biology. This seems to be true whether one is talking about a virus of green plants, bacteria, mushrooms, algae, insects, higher animals, or humans. In all cases, during the reproductive cycle the genetic material of the virus becomes a functional part of the cell it has infected. The genes added to the cell by the virus cause the infected cell either to produce more virus particles, with cell death usually the end result, or to become changed and acquire new characteristics. (Goodheart, 1969, p. 1)

A Brief History of Virology

Disease is from old and nothing about it has changed. It is we who change, as we learn to recognize what was formerly imperceptible.

Jean-Martin Charcot

The origin of infectious diseases was first attributed to supernatural and cosmic phenomena. Not until the 19th century could man take an initial glimpse into the nature of infectious agents, and indeed, only during the 20th century has a true understanding of micro-organisms as the causative agents of disease begun.

The word 'virus' derived its origin in Latin twenty-one centuries ago where it assumed the original meaning of 'poison' or 'venom' as well as a more figurative interpretation of 'virulent or bitter feelings'. The former meaning can be seen in the following quotation of about 50 A.D. from the Roman encyclopedist, Cornelius Anulus Celsus:

> Especially if the dog is rabid, the virus should be drawn out with a cupping glass. (Hughes, 1977, P. 3)

The latter occurred more frequently in nonscientific writings, as in this epigram by Martial:

> Lest you should waste your time, keep your virus for those that fancy themselves. (Hughes, 1977, p. 3)

However, despite the interpretation, 'virus' has always signified a poison in either the figurative or nonfigurative sense, and from its origin until the present has been customarily employed to denote something menacing to one's health.

Gradually, the vague idea of a 'virus' was replaced with a less ambiguous, but still undefined, interpretation. In 1840, Jacob Henle published "Von den Miasmen und Contagien", a theoretical paper in which he collated a variety of seemingly unrelated experimental findings from Agostino Bassi's work on the silkworm disease of muscardine to the studies of fermentation based on the works of Cagniard-Latour, Schwann, and Kützing. This work promoted the animate contagion theory, which held that infectious diseases were caused by minute living organisms which reproduced in the

body. Nevertheless, without direct experimental evidence no further advancements of this theory were possible.

However, by the 1880s, three investigators confirmed and established acceptance of the animate contagion as a causative agent of disease. Louis Pasteur (1822-95) was the first to experimentally disprove the theory of spontaneous generation by employing the use of sterile growth media. He further was able to prove that the different processes of fermentation were associated with various kinds of microbes. Joseph Lister (1827-1912), a noted surgeon, was the first to take Pasteur's idea of a sterile environment and to use it within the context of an operating room. He emerged with the first standard antiseptic protocol and dramatically increased the disease-free survival of his patients. Lister was also integral in the development of limited dilutions to obtain pure cultures of microbes. Through the use of sterile techniques, Robert Koch, student of Henle, developed solid media and microbial stains. He showed that the anthrax bacillus was the cause of anthrax (1876) and the tubercle bacillus the cause of tuberculosis (1882). Together these scientists irrefutably established that the etiology of an infectious disease was directly due to the constant association of a specific micro-organism with its host, which could be isolated and transferred to another host. Thus, by the 1880s, the germ theory of disease was firmly established.

Koch's postulates formalized this theory by defining strict rules for microbial disease, as follows:

1. The organism must be regularly found in the lesions of the disease.
2. The organism must be isolated in pure culture.
3. Inoculation of such a pure culture of organisms into the host should initiate the disease.
4. The organism must be recovered once again from the lesions of the host.

One cannot understand the concept of a submicroscopic organism (i.e. virus) without the elucidation and characterization of the microscopic organism. As such, it was not until the breakdown of the *postulates* that the concept of a virus could be realized for the first time. Three scientists would step forward to challenge these *postulates*. Adolf Mayer (1843-1942), while working to understand the nature of tobacco mosaic disease (TMD), unbeknownst to himself, reported the first experimental data for transmission of a viral disease when, in 1883, he showed that TMD could be transferred to healthy plants through their inoculation with the extracted juices of a diseased relative. However, he could show neither a bacterial nor fungal agent to be the cause of the disease and thus concluded that TMD was "bacterial, but that the infectious forms [had] not yet been isolated, nor [were] their forms and mode of life known." (Mayer, 1886, P. 465) Dmitri Ivanofsky (1864-1920),

upon study of Mayer's results in 1892, would repeat Mayer's observations with one added restriction. This was the use of the Chamberland filter, which had been perfected by Charles Chamberland to purify water by retarding most bacteria. Ivanofsky reported that "the sap of leaves infected with tobacco mosaic disease retains its infectious properties even after filtration through Chamberland filter candles." He then suggested that a bacterial toxin was the most likely cause of the disease:

> According to current views, the infectiousness of the filtrate can be explained most simply by assuming the presence of a poison elaborated by bacteria present in the tobacco plant and dissolved in the filtered sap. (Ivanovski, 1892, p. 69)

or even that:

> The bacteria of the tobacco plant passed through the pores of the Chamberland filter candles, even though I checked the filter...before each use and convinced myself of the absence of fine cracks and openings. (Ivanovski, 1892, p. 69-70)

The third scientist, Martinus Beijerinck (1851-1931), would play the key role in developing the concept of a virus. Unknowingly, he repeated Ivanofsky's experiment in 1898 but was also unable to determine a microbial causative. Nonetheless, he showed that even after filtration and dilution of the diseased sap, the infectious agent regained its virulence after replication in the plant and could be successfully transmitted through an unlimited number of plants. The cumulative results were paradoxical: The agent had the ability to infect and multiply within the context of

a living tissue, which suggested a micro-organism. However, it could not reproduce in the cell-free sap of the plants, was filterable and was resistant to alcohol, weak formalin, and desiccation, all of which would seem to indicate a nonliving material. In one final experiment Beirjerink attempted to prove the nature of the infectious agent. He placed sap from infected tobacco leaves onto the surface of a solidified agar medium, and after allowing ten days for any possible diffusion to occur, scraped off the top layer and injected plants with a fresh bottom layer of agar. This inoculation resulted in disease, which Beijerinck explained as follows:

> A virus [infectious agent] composed of small, discrete particles will remain on the surface because it cannot diffuse into the molecular pores of the agar plates. In this situation the deeper layers of agar will not become virulent. Conversely, a water-soluble virus will be able to penetrate a certain depth into the agar plates. (Beirjerink, 1898, p. 19)

> Although diffusion occurred over a distance of only several millimeters, nonetheless it seems to show in actual fact the virus is really liquid or dissolved and not corpuscular. (Beirjerink, 1898, p. 21)

Thus, he concluded that the cause of TMD was a living infectious agent in fluid form for which he coined the term *contagium vivum fluidum*. This concept would ignite a 25 year debate as to the true nature of the virus; liquid or particulate? Not until Felix d'Herelle developed the plaque assay in 1917, and the first electron micrographs were taken of tobacco mosaic virus in 1939, was this dispute settled and the virus defined as it is today.

Thus, the emergence of virology as an independent science did not occur until the 1940s, yet the field has been instrumental in the development and understanding of molecular biology as a whole. Although it would be an onerous task to mention all the scientists who have contributed to this field, a few pivotal experiments are worth mentioning. The first experiment was that conducted by A.D. Hershey and M. Chase in 1952. In this set of experiments Hershey and Chase helped to establish that DNA was the infectious component of bacteriophage. Thus, for the first time scientists were able to prove that DNA and not protein was the inheritable material which directs the synthesis of life. The following year, using this concept of inheritance, J.D. Watson and F.H.C. Crick first described the molecular structure of DNA and its replicative mechanism. In 1956, A. Gierer, G. Schramm, and H. Fraenkel-Conrat demonstrated that RNA is the infective component of tobacco mosaic virus. TMV RNA and its nucleotide sequence helped to confirm codon assignments for the genetic code while adding clear evidence for its universality among all life forms. Thus, the understanding of a simple submicroscopic infectious agent helped to establish some fundamental principles of a more complex eukaryotic system.

The subsequent identification and understanding of virally induced diseases led naturally to the pursuit of

medical cures. Unlike bacterial diseases, viral infections are not very susceptible to chemotherapy. Instead, the most efficient way to treat a viral disease is to prevent its establishment within the host through vaccination. The earliest example, and the one to which we owe the term 'vaccination', was Edward Jenner's cow pox vaccination which was empirically developed in 1798. With the understanding of induced host resistance through vaccination, molecular characterization of the virus, and the advent of cell culture techniques, modern vaccination programs became feasible. World-wide vaccination programs have led to one of the most remarkable achievements of our time, the eradication of smallpox, a disease with a history of over 2,000 years. Viral vaccines now include live-attenuated vaccines, killed vaccines, and subunit vaccines, and are used the world over for protection from such diseases as polio, measles, mumps, rubella, and hepatitis. However, not all viral diseases can be prevented by vaccination. One of these is the deadly human immunodeficiency virus (HIV). The first published report of acquired immunodeficiency syndrome (AIDS) was circulated only 16 years ago, in June of 1981, and a treatment which controls or eliminates this virus remains elusive. Recent World Health Organization surveys reported that HIV, predominantly transmitted sexually, has already infected up to 24 million adults, of whom at least four million have died.

The emergence of other life threatening viral diseases, such as ebola, as well as those disease that have yet to be significantly curtailed, such as respiratory syncytial virus, continue to cause socioeconomic devastation in countries where health programs have not been rigorously implemented. This indicates the necessity for continued research efforts directed within the field of virology. (For a comprehensive review of the information in this section, see Hughes, 1977.)

Viral Taxonomy

As devastating agents of disease, the paramyxoviruses, including such human pathogens as measles (MV), mumps (MuV), respiratory syncytial virus (RSV), and the human parainfluenza viruses (hPIV1, 2, 3, and 4), account for significant morbidity and mortality world wide each year. Even with the implementation of vaccination programs, the spread of measles virus in underdeveloped countries has yet to be significantly curtailed. Problems with infection from RSV and hPIV are even more pressing as one is susceptible to repeated infections. Aside from the acute diseases that these viruses cause, they have also been implicated in such chronic diseases as subacute sclerosing panencephalitis (SSPE), multiple sclerosis, Paget's disease, and chronic autoimmune hepatitis (Black, 1991; Huber et al., 1991; Collins et al., 1993; Fooks et al., 1993). In addition to their medical relevance, these viruses offer researchers the

opportunity to study and analyze many biochemical processes by serving as simple model systems for the more complex eukaryotic cell.

Sendai virus (SV) is a member of the family Paramyxoviridae, which is among six families of viruses whose linear genomes are composed of single-stranded RNA of the negative sense. The genome is contained within a helical nucleocapsid core, and enclosed within an envelope consisting of viral glycoproteins and the host cell plasma membrane. Three of these families, Paramyxoviridae, Rhabdoviridae and Filoviridae, contain non-segmented genomes, whereas Orthomyxoviridae, Arenaviridae and Bunyaviridae consist of segmented genomes of seven or eight, two, and three subunits, respectively. The Paramyxoviridae family is subdivided into three genera of viruses; the paramyxoviruses, morbilliviruses, and pneumoviruses with morbilliviruses and paramyxoviruses being more related by criteria such as nucleotide sequence conservation, gene number and order, and detailed morphology (Pringle, 1991). The Paramyxovirus genomes are remarkably similar in their size (15,000-16,000 nt), transcriptional control sequences, non-transcribed intergenic regions, and strategies for RNA synthesis and processing (Pringle, 1991). The genetic content and replication strategies of the rhabdoviruses, including vesicular stomatitis virus (VSV) are also quite similar to the paramyxoviruses (Galinski and Wechsler, 1991). Knowledge

of these viruses had been limited by the inability to mutate the viral genome directly, since the template recognized for viral RNA synthesis is exclusively a ribonucleoprotein complex. Recently, however, the advent of infectious cDNA clones of the full length genomes for rabies virus (Schnell et al., 1994), RSV (Collins et al., 1995), Sendai virus (Garcin et al., 1995), measles virus (Radecke et al., 1995) and VSV (Lawson et al., 1995; Whelan et al., 1995) will now allow for direct genetic manipulation of these negative strand RNA viruses and should promote a substantial increase in the understanding of their unique mode of replication. Since the goal of this research is to characterize the structure and function of the phosphoprotein (P) subunit of the Sendai virus RNA dependent RNA polymerase, the remaining text will focus on what is currently known about Sendai virus (SV) and the Sendai NP, P and L proteins required for RNA synthesis.

The Virion and the Viral Lifecycle

The prototypic paramyxovirus, Sendai virus, is a murine pathogen that produces a pneumonia in mice which is strikingly similar to the pathology seen with human parainfluenza type 1 (Parker and Richter, 1986). The enveloped virion contains two viral glycoproteins, the fusion protein (F) and the hemagglutinin-neuraminidase protein (HN), which mediate attachment and penetration of the virus to susceptible cells (Pringle, 1991). The matrix protein (M) is

proposed to be important for viral budding, serving as a bridge between the plasma membrane containing the viral glycoproteins and the nucleocapsid core (Peeples, 1991). The viral genome containing 15,248 nucleotides (nt) is tightly associated with approximately 2600 molecules of the nucleocapsid protein [NP-524 amino acids (aa)] which renders the helical nucleocapsid (RNA-NP or Nuc) nuclease resistant (Lamb et al., 1976; Galinski and Wechsler, 1991). It has been proposed that there is one NP protein per 6 nts, and this has been defined as the "rule of six" (Egelman et al., 1989; Calain and Roux, 1993). The nucleocapsid is approximately 15 nm in diameter and 1 μm in length (Galinski and Wechsler, 1991). The helical nucleocapsid is found in several discrete pitch states, which include the relatively tight coils with pitches of 5.3 and 6.8 nm visible in negatively stained preparations, and the extended coils with a pitch of 37.5 nm found in shadowed preparations (Egelman et al., 1989; Pelet et al., 1996). Thus, the nucleocapsid appears to be a dynamic structure, and the transitional states are proposed to have a role in the packaging of the nucleocapsid core within the virion, as well as in RNA synthesis, where the more extended form is presumed to be copied by the viral polymerase (Heggeness et al., 1981; Egelman et al., 1989). The viral RNA-dependent RNA polymerase, composed of the phosphoprotein (P-568 aa, 70 kDa) and the large protein (L-2228 aa, 240 kDa), has been shown to

be packaged within the virion with about 300 molecules of the P protein and 30 molecules of the L protein loosely associated with the nucleocapsid core (P-L-Nuc) (Portner and Murti, 1986; Galinski and Wechsler, 1991). This preassociation may serve to facilitate mRNA synthesis upon infection.

Sendai virus RNA synthesis occurs within the cytoplasm of the infected cell, and transcription by the viral polymerase initiates at the precise 3′ end of the encapsidated genome, yielding first leader RNA (*le+*, 55 nt), which terminates near the start of the first mRNA (Banerjee and Barik, 1992; Lamb and Kolakofsky, 1996). Following leader RNA synthesis, there is the sequential and polar transcription of the six major mRNAs in the following order: 3′-NP-P/C/V-M-F-HN-L-5′ such that the NP mRNA is most abundant and the L mRNA is the least abundant (Kolakofsky et al., 1991; Moyer at al., 1991). The Sendai virus mRNAs, but not leader RNA, are modified by the viral polymerase by capping and methylation at the 5′ end and by polyadenylation at the 3′ end (Galinski and Wechsler, 1991).

The P gene is unique among the paramyxovirus genes in that it encodes several nonstructural proteins in addition to the phosphoprotein, which is encoded by the longest open reading frame (ORF) beginning at nt 104. The nested set of proteins, C, C′, Y1, and Y2, are translated from the overlapping (+1) reading frame of the P mRNA by

transcriptional initiation at alternative start sites (nt 81, 114, 183, and 201, respectively). A unique feature of many paramyxoviruses has been termed mRNA editing. The V protein is made from an edited P mRNA whereby the viral polymerase adds a single guanosine at nt 1053, presumably by a stuttering mechanism similar to polyadenylation, and this causes a -1 frameshift of the P ORF during the translation of the edited mRNA (Vidal et al., 1990a, b; Pelet et al., 1991; Kolakofsky et al., 1991). The resultant V protein thus contains a unique cysteine rich carboxy-terminal end, but is amino-coterminal with the P protein from aa 1-317.

Sequence analysis has revealed a consensus sequence, 3'-UNAUUCUUUUU GAA UCCCANUUUC-5' between each of the mRNA gene boundaries. The first 11 nt represent the 3' end of the previous gene and are thought to be the putative polyadenylation signal; the next 3 nt are a nontranscribed intergenic spacer sequence conserved in all but the HN-L junction (GGG); and the last 10 nt are proposed to encode the start signal for the initiation of the next gene and possibly the capping and methylation signals (Gupta and Kingsbury, 1984; Moyer and Horikami, 1991). The exact mechanism of mRNA synthesis and processing has yet to be defined, but it has been proposed that the L subunit of the RNA-dependent RNA polymerase may play the major catalytic role, due to its large size and low level of expression within the host cell (Galinski and Wechsler, 1991; Moyer and Horikami, 1991).

The switch from transcription to replication is thought to be related to the concentration of NP protein within the cytoplasm of the infected cell, since replication, but not transcription, requires de novo protein synthesis. This was demonstrated by continued transcription and the absence of replication in the presence of cycloheximide (Carlsen et al., 1985; Galinski and Wechsler, 1991). RNA replication requires the simultaneous synthesis of the RNA and its encapsidation by NP, such that the replicative intermediates are always found to be encapsidated rendering them nuclease-resistant (Blumberg et al., 1981). It has been proposed that once the level of soluble NP (NP_0) has reached a critical concentration, encapsidation of the nascent RNA is initiated and continues in a cooperative fashion concomitantly with RNA synthesis.

Experimental evidence suggests that NP is maintained in a soluble form by the formation of the soluble P-NP_0 complex which, like the VSV P(formerly NS)-N complex, serves as the encapsidation substrate (Peluso and Moyer, 1988; Horikami and Moyer, 1992). However, it is not clear whether the NP protein or the P protein is required for recognition of a specific sequence during the initiation of encapsidation. Once encapsidation has been initiated, it is believed that the viral polymerase becomes unresponsive to the initiation and termination signals at the gene junctions and thus, RNA

synthesis continues down the entire length of the genome (Baker and Moyer, 1988; Kolakofsky et al., 1991). Replication progresses through a plus (+) strand encapsidated intermediate, the antigenome, which directs the replication of the progeny (-) strand genome. Analogous to the *le+*, a (-) leader RNA (*le-*, 57 nt) is expressed from the 3′ end of the antigenome and contains sequences up to the end of the L gene. Presumably *le-* synthesis occurs when there is an insufficient concentration of NP for progeny genome encapsidation (Leppert et al., 1979; Vidal et al., 1989; Chandrika et al., 1995).

Sequence analysis and directed mutational studies have revealed possible *cis*-acting sequences responsible for the initiation of RNA synthesis at the 3′ end of both the genome and antigenome RNA. For SV, the replication promoters are largely confined to the first 120 nt (up to the NP protein AUG) and the terminal 145 nt of the genome. The first 12 conserved and complementary nucleotides at the ends of the genomes/antigenomes (3′ duodecamer) of each *Paramyxovirinae* genus are clearly involved in the initiation of RNA synthesis (for SV, 3′ UGGUUU GUUCUC 5′). The remainder of the *le+* and *le-* regions, however, are not strongly conserved, although they are always very AU-rich. A second semiconserved sequence at nt 77-96 from the ends of several paramyxovirus genomes/antigenomes, the BB box (^{77}AR GRRCAR GTYCNA GACYYY^{96}), may also be involved in RNA synthesis (Crowley et al., 1988;

Blumberg et al., 1991). The BB box contains a majority of pyrimidine residues which is bounded by multiple sterically bulky G residues. This could provide a unique structure which could guide and/or facilitate polymerase attachment (Billeter and Cattaneo, 1991), however, there is no experimental data to support this hypothesis.

As previously stated, for Sendai virus each NP subunit of the nucleocapsid is associated with precisely 6 nt and it appears that not only does the primary sequence of the RNA play a role, but the hexamer phasing of these sequences is important as well (Pelet et al., 1996; Tapparel and Roux, 1996). However, this is not true of RSV, VSV, or SV5. The hexamer phase presumably results because the position of each nucleotide within a given subunit is different from each other within the subunit (i.e. positions 1 through 6), but is the same between the same positions of different subunits (i.e. position 1 of two different NP molecules). Therefore, the *cis*-acting promoter sequence(s) is viewed by the viral polymerase not only as a linear sequence(s), but as one(s) in which the nucleotides are grouped as hexamers. Pelet et al. (1996) have proposed that the polymerase is able to make multiple base contacts over at least 78 nt or 13 NP subunits, which is equal to one turn of the nucleocapsid helix.

Once the replication cycle is complete, the viral nucleocapsids associate with the viral M protein. It is believed that the basic M protein (+14 and +17 at neutral pH)

is attracted to the acidic nucleocapsid (Morgan et al., 1985; Galinski and Wechsler, 1991). The M protein thus positions the nucleocapsids beneath the plasma membrane containing the glycoproteins through the M protein's simultaneous interactions with the cytoplasmic tails of the F and HN proteins (Galinski and Wechsler, 1991). However, the exact mechanism is unknown. Once these interactions are complete the viral particles are released by budding through the plasma membrane.

Defective Interfering Genomes

Defective interfering (DI) particles of Sendai virus have been utilized extensively in both *in vitro* and *in vivo* replication assays (Horikami et al., 1992; Curran et al., 1993). DI particles are essentially mutant viruses containing extensive deletions of the coding sequence and thus require helper Sendai virus to provide viral proteins for replication. However, the DI also interferes with helper virus replication (Re, 1991). The SV DI particles are classified according to the nature of their 3′ and 5′ ends. DIs with inverted repeats of the 5′ end are referred to as the copy-back (or panhandle) class of DI RNA. These DI genomes contain at the 5′ end the C-terminal portion of the L gene including the polyadenylation signal, the intergenic trinucleotide, and the 57 base leader sequence of the 5′ genomic end and the 3′ end is the complement of the 5′ end

(Re, 1991). DIs which retain their authentic 3′ and 5′ ends, but contain extensive internal genomic deletions, are referred to as internally deleted DIs. These DI genomes have been shown to contain the signals for both transcription and replication and are able to produce authentic mRNAs (Engelhorn et al., 1993). The copy-back class of DI RNA is predominant in nature, where its replication exceeds that of the wild type (wt) SV genome.

The DI particles replicate in a manner similar to wt SV. Recently, the mechanism of DI interference has been investigated. For VSV it was shown that the terminal complementarity of the copyback genome directs increased replication. Therefore, it is understandable how the 5′ copy back DI, due to its terminal complementarity (110 nt) and small size, has a replicative advantage over the wt genome (Wertz et al., 1994). For Sendai virus, however, it appears that the feature most important for replication efficiency is contained within the promoter primary end sequences, specifically nt 1-31 of the DI antigenome 3′ end, and is not due to the degree of terminal complementarity (Tapparel and Roux, 1996). For Sendai virus, a plasmid containing a cDNA clone of the (-) DI-H genome (1410 nt) has become available, and when coexpressed with the proteins necessary for RNA synthesis, P, L, and NP, one is able to generate nuclease resistant replicative products that mimic the genome amplification of a wt infection *in vivo* (Calain et al., 1992;

Calain and Roux, 1993; Curran et al., 1993). This clone should prove invaluable in the full delineation of template function.

Viral Proteins Required for RNA Synthesis

The NP Protein

The NP protein is an essential component of both protein-protein, including NP-NP, NP_0-P, and P-L-Nuc, and protein-RNA interactions necessary for viral replication (Horikami et al., 1992; Buchholz et al., 1993; Curran et al., 1993). Tryptic digestion of tightly coiled nucleocapsids, identified a 48 kDa N-terminal cleavage product, which remained associated with the viral RNA, and a 12 kDa C-terminal cleavage product which was no longer associated with the nucleocapsid (Heggeness et al., 1981). Upon inspection by electron microscopy (EM), it was shown that the digested nucleocapsids appeared morphologically similar to the native nucleocapsids and that the associated RNA remained resistant to nuclease treatment. These data suggested that the domains of NP responsible for assembly of a nuclease resistant helical nucleocapsid lay in the N-terminal 80% of the protein. Such domains would be responsible for both NP-NP and NP-RNA interactions. These data also suggested that the C-terminal 20% of the protein is exposed on the surface of viral nucleocapsids and is not involved in NP-NP self-assembly or in binding to the RNA. Epitope mapping with

monoclonal antibodies revealed that the majority of the antibodies recognized epitopes from aa 425-524, which is consistent with the idea that the C-terminus lies on the surface of the nucleocapsid (Deshpande and Portner, 1984; Gill et al., 1988; Fisher, 1990).

To better define regions of NP involved in its varying interactions, a panel of 22 deletion mutants covering the entire NP protein were constructed and tested for self-assembly, RNA replication both *in vitro* and *in vivo*, NP_0-P complex formation, and P-Nuc complex formation (Buchholz et al., 1993, 1994; Curran et al., 1993). The summary of these data suggest that the first 400 aa are required for both self-assembly of NP and *in vitro* replication as well as NP_0-P interactions. The fact that the self-assembly domains and NP_0-P domains appear to overlap presents a model which allows NP to self-assemble on the RNA only when released from the P protein. P thus serves to keep NP in a soluble, monomeric state prior to RNA encapsidation.

Although the C-terminal 124 aa were shown not to be required for these functions, they were shown to be necessary for replication *in vivo*, which is a measure of template function (Curran et al., 1993). Deletions of aa 426-524 abolished the ability of P to bind to the nucleocapsid template (P-Nuc) (Buchholz et al., 1994). As P is responsible for the assembly of the polymerase complex on the

nucleocapsid (P-L-Nuc) (Portner et al., 1988; Horikami and Moyer, 1995), these data are consistent with the inability of the C-terminal deletion mutants to support *in vivo* replication, and thus to serve as a template for polymerase assembly. Further support for a C-terminal P-Nuc binding site was provided by Ryan et al. (1993) who reported that a monoclonal antibody mapping to aa 405-524 released P protein from nucleocapsids as well as prevented exogenous P protein from binding to nucleocapsids *in vitro*.

Recent work by Myers et al. (1997a, b) has attempted to better define functional domains within the amino-terminal 400 aa of NP through the construction of various amino acid point mutations and the use of fusion protein constructs. Using clustered charge-to-alanine mutagenesis in a highly charged region of NP from aa 107 to 129, three mutants, NP114, NP121, and NP126, which were shown to be wild type in protein-protein interactions, including NP_0-P, NP-NP, and P-L-Nuc, and *in vitro* replication, were unable to serve as templates for further amplification *in vivo* (Myers et al., 1997a). Thus it is proposed that amino acids 114 to 129 of the NP protein, as well as the C-terminus of the protein, are required for the nucleocapsid to function as a template in viral genome replication.

In order to define putative NP-NP binding domains required for nucleocapsid assembly, Myers et al. (1997b)

constructed fusion proteins between the monomeric maltose-binding protein (MBP) and portions of NP. Fusion proteins which contained the central conserved region (CCR), a highly conserved hydrophobic domain, (amino acids 258-357, MBP-NP1) and the N-terminal 255 amino acids (MBP-NP2) of NP both oligomerized, suggesting that these regions are important for NP-NP self-assembly. Also MBP-NP1 functioned as an inhibitor of replication. Site-directed mutagenesis further identified Phe 324 to be essential for the NP-NP interaction. Another mutation at Tyr 260 conferred the ability of the mutant NP protein to self-assemble, but disrupted the usual helical structure of the nucleocapsid as demonstrated by EM. These irregularly assembled particles also appeared to lack any associated RNA. Two mutants containing changes at L299 and I300 and I313, in contrast, appeared to form all of the appropriate protein interactions, but were inactive in replication, suggesting that NP-RNA interactions were disrupted. In summary, these data identified residues in the CCR which appear to be important for NP-NP or NP-RNA interactions and for genome replication.

The L Protein

The L protein subunit of the RNA dependent RNA polymerase is believed to contain the majority of the catalytic activities for RNA synthesis, including polymerization, mRNA capping and methylation, and polyadenylation. The methylation and polyadenylation

activities have been previously identified in the analogous VSV L protein (Hammond and Lesnaw, 1987; Hercyk et al., 1988; Moyer and Horikami, 1991; Hunt and Hutchinson, 1993). The L gene is the most conserved among the paramyxovirus and rhabdovirus genes. Sequence alignment among these various L genes has revealed six conserved domains (I-VI) within the protein which are hypothesized to represent functional domains (Poch et al., 1990; Sidhu et al., 1993). Of importance, Domain III (aa 758- aa 989) contains the putative template recognition and polymerization site. This site contains the conserved peptide sequence GDN which has homology to the conserved GDD catalytic domain of other RNA dependent RNA polymerases (Jablonski et al., 1991). Direct mutation of these residues within the rabies virus, bunyamwera virus, and VSV L proteins has shown this conserved sequence to be critical for RNA synthesis (Sleat and Banerjee, 1993; Jin and Elliot, 1992; Schnell and Conzelmann, 1995).

Studies aimed at dissecting the various functional roles of the L protein have been limited. Complementation experiments have shown inhibition of RNA synthesis to be linked to temperature sensitive mutations arising within the L gene (Pringle, 1991). In another mutant, a temperature sensitive defect and an aberrant polyadenylation phenotype were also mapped to the L gene (Hunt and Hutchinson, 1993). At the amino acid corresponding to the polyadenylation

phenotype within Sendai L, mutations were constructed which presented multiple defects in RNA synthesis, but not the polyadenylation defect (Horikami and Moyer, 1995). An intrinsic kinase activity has also been associated with the L protein, which has been hypothesized to play a role in phosphorylation of the P protein and thus, the regulation of polymerase function (Sanchez et al., 1985; Hammond et al., 1992; Einberger et al., 1990).

Structural studies aimed at dissecting the protein-protein interactions of L have also been limited. Of relevance, deletion mutants of various L proteins have mapped the homologous P protein binding domains to the amino-terminal 1247 aa of the SV5 L protein, the amino-terminal 408 aa of the MV L protein, and the amino-terminal 1147 aa of the Sendai virus L protein (Parks, 1994; Horikami et al., 1994; Chandrika et al., 1995). Recently, the Sendai L protein has been shown to interact with the C protein (Horikami et al., submitted). Possible interactions between other viral proteins or cellular proteins have yet to be identified.

The P Protein

The RNA dependent RNA polymerases of negative-strand RNA viruses are unique in that they recognize the viral genome only in an encapsidated form. The Sendai virus RNA polymerase complex consists of two viral proteins, P and L, which must be coexpressed in order to form an active enzyme (Horikami et al., 1992). The L protein is believed to

contain most of the catalytic activities necessary for viral RNA synthesis and processing, although there presently is little direct experimental data to support this hypothesis. The precise function of the P protein within the polymerase complex is unknown. So far it has not been shown to contain any catalytic function, but due to its high degree of phosphorylation it has been proposed to act as a regulatory factor (Kolakofsky et al., 1991) or possibly to function as a processivity factor for RNA elongation, as was postulated for VSV (De and Banerjee, 1985). Alternatively or in addition, the P protein is believed to be important for proper conformational folding in the stable expression of the L protein. Pulse-chase experiments show L must be coexpressed with P to be stable (Horikami et al., 1997). Binding of the P-L complex to the nucleocapsid template occurs through the P protein subunit of the polymerase (Portner et al., 1988, Horikami et al., 1995).

Sequence alignment of P proteins has shown them to be the most divergent of the nucleocapsid-associated proteins, and, as a group, probably the least similar of all of the paramyxovirus proteins, varying in size from 241 aa (RSV) to 602 aa (PIV3) (Morgan, 1991). This variability has made it difficult to interrelate experimental evidence about the different P proteins. Several approaches have been taken in the analysis of Sendai P protein function and the domains required for P-L, P-NP_0, P-Nuc, and P-P interactions,

including immunogold labeling (Portner and Murti, 1986); monoclonal antibody epitope mapping (Deshpande and Portner, 1985); topography analysis of phosphate residues (Hsu and Kingsbury, 1982); identification of protein complexes required for *in vitro* SV-DI-H replication (Horikami et al., 1992); deletion analysis (Ryan and Kingsbury, 1982; Ryan and Portner, 1990; Smallwood et al., 1994; Curran et al, 1994, 1995) and phosphorylation studies (Byrappa et al., 1995, 1996).

Studies aimed at defining the specific biological activities of the P protein in RNA synthesis have been important in assigning a role for P as a subunit in the active viral polymerase. Portner et al. (1986) have shown by immunogold staining with monoclonal antibodies that the distribution of P in nucleocapsids derived from the cytosol differed from the distribution of P originating from virions. Cytoplasmic nucleocapsids showed P molecules to occur in 4-10 discreet clusters at varying locations along the length of the nucleocapsid. However, virion derived nucleocapsids showed P to be uniformly distributed over the entire length of the genome. These data suggest that upon release of the nucleocapsid into the host cell cytoplasm, the associated polymerase complexes become active and are localized to their sites of action.

Epitope mapping showed that antibodies directed against P mapped to three antigenic sites, that these antibodies

inhibited viral RNA synthesis, and that the antibodies all recognized determinants within the carboxyl-terminal 40% of the protein (Deshpande and Portner, 1985; Ryan et al., 1991). *S. aureus* protease V8 digestion of template associated P protein generated an attached 40 kD protein, which could still function in mRNA synthesis and mapped to the carboxy-terminus of the protein (Chinchar and Portner, 1981). All of these data are consistent with P being an essential component of the viral RNA polymerase and suggest that the domains necessary for RNA synthesis are located in the C-terminus of the protein.

The availability of an *in vitro* Sendai DI-H replication assay using proteins expressed from cloned genes has made it possible to show that two protein complexes, P-L and P-NP_0, are required for *in vitro* Sendai virus RNA replication (Horikami et al., 1992). Furthermore, the coexpression of the proteins in each complex was required for activity. Direct interaction between P and L and P and NP was shown by coimmunoprecipitation assays using monoclonal antibodies against P and NP, respectively. These data suggest that P is required not only for P-L polymerase activity, but also for use as an encapsidation substrate, probably to keep NP_0 in a soluble form (Horikami et al., 1992; Curran et al., 1995).

A third possible role for P protein in RNA synthesis has recently been described. Curran (1996) provides evidence

that while part of the template associated P is bound as a P-L complex, the rest is bound to the template independently of L, and this latter form of P is also essential for mRNA synthesis. Although the exact mechanism of this supplemental function has yet to be delineated, these results are consistent with previous evidence which reported that optimal *in vitro* transcription required a molar excess of P protein greater than that which is required for polymerase complex formation (Thomas et al., 1985; De and Banerjee, 1985; Canter et al., 1993; Curran, 1996).

Deletion analysis has further delineated regions of the Sendai P protein important for various protein-protein interactions. Deletions along the entire length of the P protein were constructed and tested by several assays to yield the model of important domains: 1. The nucleocapsid (Nuc) binding domain has been assigned to two non-contiguous regions, aa 345-411 and aa 479-568 (Ryan and Portner, 1990; Ryan et al., 1991). 2. The encapsidation substrate ($P\text{-}NP_0$) interaction domain has been assigned to aa 33-41 and aa 479-568 (Curran et al., 1995) and we have recently shown that sequences between aa 78-316 are also involved (Horikami et al., 1996). 3. The L binding domain on P has been mapped to aa 412-445 (Smallwood et al., 1994; Curran et al., 1994) between the Nuc binding sites. 4. An acidic activation-like domain necessary for mRNA synthesis has been mapped to aa 1-77, with a redundant activation-like domain mapping to aa 78-114 (Curran et al., 1995).

More recently the P protein has been shown to oligomerize forming a homotrimeric complex. Epitope dilution assays and deletion analyses were able to localize the P oligomerization domain to aa 344-411 (Curran et al., 1995; Horikami et al., 1996). Computer analysis predicts that this domain has a coiled coil structure which is important for the P-P interaction. This oligomerization domain overlaps the most amino terminal Nuc binding domain and it has been proposed that the different protein-protein interactions necessary to form the P-Nuc and P-P interactions occur on different faces of the coiled coil. However, the data are also consistent with the model where P oligomerization is required for the P-Nuc interaction, such that deletion of this domain would disrupt oligomerization and thus, interfere with binding to the nucleocapsid. The exact function for P oligomerization has yet to be defined, and it is unknown which form of the P protein is present in complexes with each of the other viral proteins.

Studies aimed at defining the role of phosphorylation in Sendai P protein function have been limited and much of what is known is modeled on studies of the P proteins of other negative strand RNA virus families. Although the SV P protein represents only 10% of the virion protein mass, it contains 40% of the virion protein-bound phosphates and is thus the most highly phosphorylated viral protein on a mole-

per-mole basis (ca. 5-6 moles phosphate/ molecule P protein) (Hsu and Kingsbury, 1982). Several studies of phosphorylation revealed that a phosphate-rich, 18 kD cleavage product mapped to the amino terminal 25% of the SV P protein and contained approximately 80% of protein associated phosphates (Hsu and Kingsbury, 1982). In contrast to these data, the use of the virion associated protein kinase (VAPK)-phosphorylated P protein, partial V8 proteolysis, and a panel of monoclonal antibodies showed that the major phosphorylation site mapped to the second quarter of the protein (Vidal et al., 1988).

Only very recently has the exact site of the P phosphorylation been mapped. Through studies involving tryptic phosphopeptide mapping of P protein from virus-infected cells and purified virions, the major phosphorylated residue has been determined to be S249 (Byrappa et al., 1995, 1996). Studies of cell free phosphorylation of P revealed a strikingly dissimilar pattern of phosphorylation. It has thus been proposed that phosphorylation is due to a cellular kinase *in vivo*. Sequence analysis revealed a possible consensus site for phosphorylation by a proline-directed protein kinase (SP), since mutation of P250 to alanine was found to be necessary for S249 phosphorylation (Byrappa et al., 1996). Although the V8 protease data suggest this domain is not essential for RNA synthesis (Chinchar and Portner, 1981), it is important to stress that those studies

were carried out with P protein which was already assembled onto the template. Therefore, the phosphorylation of P may still prove to be essential for complete P function. In order to assess possible functions for this phosphorylation we must turn our attention to what is known about the requirements of phosphorylation for the P proteins of other similar viruses.

Much of our current thinking on the relevance of phosphorylation has been derived from studies of the VSV and RSV P proteins. For VSV it has been shown that S60 and T62 are the major phosphorylation sites. Phosphorylation is constitutive, is directed by cellular casein kinase II and is absolutely required for RNA synthesis (Barik and Banerjee, 1991, 1992; Beckes and Perrault, 1992; Gao and Lenard, 1995a, b; Das et al., 1995). It was also shown that S236 and S242 were phosphorylated by VAPKs, but recently these phosphorylation events were shown not to be required for P function (Chattopadhyay and Banerjee, 1987; Beckes and Perrault, 1992; Gao and Lenard, 1995b). These data were obtained through analysis of phosphate-free P protein (P^0) overexpressed in *E. coli*. Primary phosphorylation of P^0 by casein kinase II is necessary and sufficient to generate a functionally active P protein (P^1). However, P^1 is able to serve as a substrate for further phosphorylation (P^2) by VAPKs albeit these are seemingly non-essential.

Further analysis has shown that the phosphorylation at S60 and T62 is necessary for P protein oligomerization, and

assembly of the P-L polymerase complex onto the nucleocapsid template (Gao and Lenard, 1995a, b). However, there is a debate as to whether a homodimeric or homotrimeric structure is formed (Gao and Lenard, 1995a, b; Das et al., 1995). Circular dichroism and biochemical studies have also revealed that phosphorylation of P^0 to P^1 significantly increases the predicted α-helical structure of the P^1 protein from 27 to 48% which indicate this structure is important for the P-P interaction (Das et al., 1995). Studies involving RSV P have shown that casein kinase II phosphorylation of S232 and S237 are absolutely required for transcriptional activity (Mazumder et al., 1994; Mazumder and Barik, 1994; Barik et al., 1995), while studies of hPIV P showed that its phosphorylation by cellular protein kinase C isoform ζ is essential for activity (De et al., 1995). All of these data reinforce the idea that phosphorylation of the P proteins of various negative-strand RNA viruses is important for their function.

The structural and functional characterization of the Sendai virus P protein is still ongoing and the exact role(s) of P in viral RNA synthesis is unknown. However, these studies indicate avenues which may be pursued in the further characterization of this protein. For our studies we will attempt to map the specific residues important for the P-L protein-protein interaction as well as to determine the role of P protein phosphorylation in biological activity.

CHAPTER 2
MATERIALS AND METHODS

Cells and Viruses

Human lung carcinoma cells (A549 cells, American Type Culture collection) were grown and maintained as monolayers at 37°C with 5% CO_2 in Eagle's minimal essential medium (F11, GIBCO BRL) supplemented with 8% fetal bovine serum (FBS, GIBCO BRL), 1 mM sodium pyruvate (100 mM, Mediatech), 2 mM L-glutamine (200 mM, Mediatech), 1% penicillin-streptomycin (pen/strep; 5000 I.U./ml and 5000 mcg/ml, respectively, Mediatech), and 1% non-essential amino acid solution (100X, Mediatech). Maintenance of African green monkey kidney cells (Vero, American Type Culture collection) was as above except for supplementation with 5% FBS.

Wild type (wt) Sendai virus (SV, Harris strain) or Sendai virus defective interfering particle (DI-H, Harris strain) were propagated in the allantoic fluid of 9-day old embryonated chicken eggs which were maintained at 37°C in an egg incubator and automatically turned every 30 minutes. The amount of Sendai virus that was used to inoculate the eggs was empirically determined, as was the ratio of Sendai virus wt and DI-H used for co-infection. Virus was grown at 33°C at 85% humidity for 3 days. 16 h prior to harvesting the

allantoic fluid, the eggs were placed at 4°C and then at -20°C for 10 min. The collected fluid was clarified by centrifugation at 2000 rpm for 10 min at 4°C in an International Equipment Company (IEC) Centra-8R centrifuge, and the supernatant stored in aliquots at -70°C.

Wt or DI-H viruses were purified by pelleting through 15 ml of 25% (v/v) glycerol in HNE buffer (10 mM HEPES, [*N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid], pH 7.4, 100 mM NaCl, 1 mM ethylenediamine tetraacetate [EDTA]) at 26,000 rpm for 5 h at 4°C in a SW28 rotor (Beckman) and L8-55 ultracentrifuge. The supernatant was decanted and the viral pellet resuspended in 1 ml ET buffer [10 mM Tris(hydroxymethyl)aminomethane-hydrochloride (Tris-HCl), pH 7.4, 1 mM EDTA] plus 10% dimethylsulfoxide (DMSO) (ET/DMSO). The virus was sonicated twice for 20 sec each and purified by banding on 7-60% (w/w) sucrose gradients in HNE buffer at 24,000 rpm for 17 h at 4°C in a SW41 rotor (Beckman). The visible virus bands were collected, diluted with ET buffer, and pelleted in a SW41 rotor at 30,000 rpm for 2 h at 4°C. The virus pellet was resuspended in ET/DMSO, aliquoted, and stored at -70°C. The protein concentration was determined using the Bradford method as outlined in *Current Protocols in Molecular Biology* (Ausubel et al., 1987).

For the generation of polymerase-free nucleocapsid templates, 4 ml of purified virus (1 mg/ml) was mixed with 4 ml of 2X High Salt Solubilizer [HSS; 2 M NH_4Cl, 20 mM HEPES,

pH 8.5, 10% (v/v) glycerol, 3.7% Triton X-100, 1.2 mM dithiotreitol (DTT)]. Following disruption of the viral particles by incubation for 2 h at 4°C, the mixture was layered in 2 ml aliquots over 2.5 ml 30% (v/v) glycerol in 10 mM HEPES, pH 8 and pelleted at 50,000 rpm for 90 min at 4°C in a SW55 rotor. The supernatant was decanted, the pellet resuspended in 2 ml total of 1X HSS buffer and incubated for 30 min at 4°C. The suspension was layered over 2 20-40% (w/w) CsCl linear gradients and the nucleocapsids banded at 36,000 rpm for 16 h at 4°C in a SW41 rotor. The visible band was collected, diluted to 5 ml with ET, and pelleted at 50,000 rpm for 90 min at 4°C in a SW55 rotor. The pellet was resuspended in ET/DMSO, and after the protein concentration was determined by the Bradford method, the nucleocapsids were diluted to 1μg/μl, aliquoted and stored at -70°C.

Recombinant vaccinia virus engineered to express the phage T7 RNA polymerase (VVT7) (Fuerst et al., 1986), provided by Dr. Edward Niles (Suny; Buffalo, N.Y.), was grown in Vero cells.* Vero cells were grown in ten 15 cm dishes to confluency at 37°C and subsequently infected with VVT7 at a multiplicity of infection (m.o.i.) of 0.05 pfu/cell. Four days post-infection (p.i.), the cells were scraped into the medium with a rubber policeman. The cells and extracellular virus were pelleted in a J10 rotor (Beckman) at 7000 rpm for 30 min at 4°C. The pellets were resuspended in 10 ml phosphate buffered saline (PBS; 1.5 mM potassium phosphate

monobasic, 4.3 mM sodium phosphate, 137 mM NaCl, 2.7 mM KCl, pH 7.2) supplemented with 1% pen/strep (PBS + P/S) and the cells were disrupted by 2 cycles of freeze/thawing followed by sonication until a uniform suspension was obtained. The titer was determined by plaque assay of serial dilutions of the viral suspension on A549 cells. The virus was stored at -70°C.

Antibodies

Immunoprecipitation and immunoblot assays utilized the following primary antibodies: rabbit anti-Sendai virus antibody (α-SV), rabbit anti-SV L (α-L) antibody specific for the SV L protein (Smallwood et al., 1994), monoclonal antibody to the Sendai L protein (Einberger et al., 1990; Curran, 1996), monoclonal antibody to the Sendai P protein (α-PM53, provided by Dr. A. Portner, St. Jude Children's Hospital, Memphis, TN) (Ryan et al., 1991), rabbit polyclonal antibody against SV P peptide fragments corresponding to aa 274-298 and 453-477 (α-P peptide, provided by Dr. K. Gupta, Chicago, IL) (Byrappa et al., 1995), and rabbit anti-glutathione S-transferase antibody (α-GST) (Chandrika et al., 1995). Secondary antibodies utilized are as follows: goat anti-mouse immunoglobulin G antibody (α-IgG, Fisher), goat anti-mouse immunoglobulin M antibody (α-IgM, Fisher), goat anti-rabbit IgG-alkaline phosphate conjugated antibody (α-IgG-AP, Fisher), and goat anti-rabbit IgG-horseradish peroxidase conjugated antibody (α-IgG-HRPO, Fisher).

Plasmids

The plasmids pGEMSV-P/C, pGEMSV-L, and pGEMSV-NP containing the SV phosphoprotein and C protein (P/C), large (L), and nucleocapsid (NP) genes, respectively, (Curran et al., 1991) were provided by Dr. D. Kolakofsky (Geneva, Switzerland). The plasmids pBSMV-P/C and pBSMV-L (Horikami et al., 1994) containing the measles virus (MV) phosphoprotein (P/C) and large (L) genes, respectively, were provided by Dr. W. Bellini (CDC; Atlanta, GA) and Dr. M. Billeter (Zurich, Switzerland). The plasmid pGEMMV-P/C was constructed by subcloning the MV P/C gene into pGEM3 by Dr. R. Chandrika (Gainesville, FL). The plasmid pTM1GSTSV-P and pTM1GSTMV-P containing the glutathione S-transferase (GST) gene (Stein et al., 1989) fused in frame to the N-terminus of the SV P and MV P genes, respectively, (Bankamp et al., 1996) were subcloned into pBSKS+ vectors by S. Smallwood (Gainesville, FL). All of the viral genes were cloned downstream of the phage T7 promoter.

Probes

Leader RNA analysis by Northern blot utilized a riboprobe (145 nt) complementary to the genomic leader RNA and the 5′ end of the NP mRNA (Vidal and Kolakofsky, 1989). The riboprobe was a uniformly ^{32}P-CTP labeled T7 transcription product of BamHI-linearized pGEMSV*l*-NP. PGEMSV*l*-NP was constructed by digestion of SP65*l*-NP with EcoRI and HindIII and subcloned into PGEM3 at the same sites.

Construction of Phosphoprotein Mutants

Sendai and Measles Virus Phosphoprotein Chimeras

P plasmids with chimeras of the SV P/C and MV P/C genes were created by A. Oberdorf (Gainesville, FL). Briefly, chimera pGEMP-AO2 was generated using pGEM1-PΔDA which contains a deletion within the SV-P gene corresponding to amino acids 412-478 at an engineered SstI site (Smallwood et al., 1994). A 215 nt polymerase chain reaction (PCR) product with SstI linkers was generated from pBSMV-P/C from nt 1086-1301 using the following set of primers: 5′ TTATGAGCTC TTGAAGGGAG AAGTTGAGTC 3′ and 5′ TTATGAGCTC GGGTTTCTTG AGAACTTCG 3′ and was cloned in frame into the deletion site of pGEM1-PΔDA with SstI to generate chimera pGEMP-AO2, which encodes PAO2, a 574 aa polypeptide. For subcloning pGEMP-AO2 to create pBSGSTP-AO2, an XhoI-EcoRI DNA fragment from nucleotides (nt) 1130-1822 of pGEMP-AO2 was substituted for the wild type sequence in pBSGSTSV-P/C at the same restriction sites.

Chimera pBSP-AO3 was engineered by first creating a StyI deletion within pBSMV-P/C, which removed nt 1025-1320 (pBSMV-PΔStyI) (Bankamp et al., submitted). A 202 nt PCR product with StyI linkers was generated from the SV P/C gene from nt 1335-1536 using the following set of primers: 5′ CTCTCCTTGG CTGAGTATCA GAAAGAACAG AACTCATTGC 3′ and 5′ CTCTCCTTGG GGTTTGTACT TCATATCTTC TAGGGTCTCC 3′ and was cloned in frame into pBSMV-PΔStyI at the StyI site. The resulting plasmid,

pBSP-AO3, encodes PAO3, a 498 aa polypeptide. For subcloning pBSP-AO3 to create pBSGSTP-AO3, a HindIII-XhoI DNA fragment from nt 676-1657 was substituted for the wild type sequence in pBSGSTMV-P/C at the same restriction sites.

Charge-to-Alanine Site Directed Mutants

Charge-to-alanine mutagenesis targeted the putative SV L binding domain of the SV P gene from aa 408 to 473 (Smallwood et al., 1994; Curran et al., 1994), generating 11 mutants containing 1 to 4 alanine substitutions each as shown in Table 1. Mutants were created either by using the Sculptor oligonucleotide-directed *in vitro* mutagenesis system version 2.1 kit (RPN 1526, Amersham) per manufacturers protocol, or by PCR directed site-specific mutagenesis (Higuchi et al., 1988). To screen putative mutants, each mutagenic primer was designed so as to introduce a new silent restriction endonuclease site. The mutagenic oligonucleotides used are shown in Table 2 with the introduced restriction sites underlined. Mutants P2, P3, P4, P5, P408/9, and S426A were made using the Sculptor kit. This protocol requires the use of single-stranded (ss) phagemid DNA and a single mutagenic oligonucleotide, so it was necessary to use pBSSV-P/C (Stratagene) which could generate the ss DNA. An EcoRI-NdeI digested fragment from nt 1130-1717 containing the mutation site was then subcloned by substitution for the wild type sequence into pGEMSV-P/CΔEcoRI at the same restriction sites for the expression studies. PGEMSVP/CΔEcoRI was constructed

Table 1. Charge-to-alanine mutant Sendai virus phosphoprotein genes and proteins

Mutant	Nucleotide Change[a]	Amino Acid Change[b]
P408/9	T1217**C**, A1221**G**, A1222**C**, A1224**G**, G1225**C**	K408**A**, R409**A**
P1	A1234**C**, A1242**G**, A1243**C**, A1244**C**, A1246**C**, A1247**G**	E412**A**, K415**A**, E416**A**
S419A	T1254**G**	S419**A**
S426A	T1275**G**, A1280**G**, T1283**G**	S426**A**
P2	A1297**C**, A1299**G**, G1300**C**, A1308**G**, A1309**C**	D433**A**, R434**A**, K437**A**
P3	A1356**G**, A1357**C**, A1362**G**, A1363**C**, A1364**G**, A1366**C**, G1367**C**, A1371**G**, A1372**C**	K453**A**, K455**A**, E456**A**, K458**A**
K453A	A1356**G**, A1357**C**	K453**A**
P455/456	A1362**G**, A1363**C**, A1366**C**	K455**A**, E456**A**
P4	A1377**G**, A1378**C**, A1386**G**, G1387**C**, A1393**C**	K460**A**, R463**A**, D465**A**
P5	A1405**C**, G1406**C**, A1414**C**, A1415**C**, A1417**C**, T1418**G**	E469**A**, D472**A**, E473**A**

[a] Nucleotide changes are listed by the wt nucleotide, its position in the sequence, and the nucleotide to which it was changed in boldface characters.
[b] Amino acid changes are listed by the wt amino acid, its position in the protein, and the amino acid to which it was changed in boldface characters.

Table 2. Oligonucleotide primers for charge-to-alanine mutations

Mutant	Mutagenic primers[a]	Enzyme
P408/9	SM203(+)-CGGATATCTACGCGGCATTCTCTGAG	EcoRV
P1	SM159(+)-CTCTGCGTATCAGGCCGCGCAGAACTC	BsoFI
	SM189(-)-GAGTTCTGCGCGGCCTGATACGCAGAG	
S419A	SM202(+)-GAACAGAACGCATTGCTCATGTCCAACC	NlaIII
	SM333(-)-GGTTGGACATGAGCAATGCGTTCTGTTC	
S426A	SM201(+)-CCAACCTAGCTACGCTGCATATCATCAC	BsoFI
P2	SM160(+)-CATCACAGCTGCAGGTGGCGCGACTGACAAC	BsoFI
P3	SM161(+)-CCTCCGTTTTTGCAGCATCAGCGGCCACCGCGACTAAGGC	BsoFI
K453A	SM329(-)-CCTCCGTTTTTGCAGCATCAAAAGAGAACAAGACTAAGGC	BsoFI
	SM338(-)-GCCTTAGTCTTGTTCTCTTTTGATGCTGCAAAAACGGAGG	
P455/6	SM330(+)-CCTCCGTTTTTGCAAAATCAGCAGCGAACAAGACTAAGGC	BsoFI
	SM339(-)-GCCTAAGTCTTGTTCGCTGCTGATTTTGCAAAAACGGAGG	
P4	SM162(+)-CAAGACTGCGGCTACCGCGTTTGCCCCATC	BsoFI
P5	SM163(+)-CCATCTATGGCCACCCTAGCCGCGATGAAGTACAAACCG	BsoFI
	Standard outside primers[a]	
	SM188(+)-CTGCTCAAGAATTCGAG	
	SM190(-)-GATCTCGAGCCCGGGATCTAGTTG	XhoI

[a] The oligonucleotide sequences are written 5′ to 3′ and the plus and minus symbols following the primer number refers to the messenger sense (+) or the genomic sense (-) of the oligonucleotide. The underlined sequence indicates the introduced silent restriction enzyme site shown on the right.

so as to remove a second EcoRI site located in the multiple cloning region upstream of the SV-P open reading frame (ORF). PGEMSVP/CΔEcoRI was created by digestion of pGEM3 with EcoRIto linearize the plasmid, blunting of the EcoRI 3'overhanging ends with T4 DNA polymerase, and religation of the plasmid DNA yielding pGEM3ΔEcoRI. Wt SV-P was digested out of pBSSV-P with Pst and Sst and cloned directionally into pGEM3ΔEcoRI at the same restriction sites to create pGEMSVP/CΔEcoRI.

The methodology for the generation of ss DNA utilizing a helper phage was adapted from the Sculptor™ kit recommendations. M13KO7 helper phage, provided by R. Condit (Gainesville, FL), was used to generate a high titer stock of M13KO7 (2.2 x 10^{10} pfu/ml) provided by T. Myers (Gainesville, FL). Ss DNA was produced as follows: early log phase cultures (6 ml, OD_{600} ~ 0.200) of *E. coli* TG1 cells [*K12Δ(lac-ProAB) Sup* E *thi hsd*Δ5/F' *tra*D36 *pro*AB+ *lac*Iq *lacZ*ΔM15] containing pBSSV-P/C in Luria broth (1% bacto-tryptone, 0.5% bacto-yeast extract, 1% NaCl) plus 100 μg/ml ampicillin (D[-]-α-Aminobenzyl-penicillin, Sigma) (LB + amp) were infected with M13KO7 at an m.o.i. of 10 and incubated in an air shaker at 200 rpm for 1.25 h at 37°C. The cells were then diluted to an OD_{600} < 0.200 with LB + amp and supplemented with 70 μg/ml kanamycin A (Sigma). The cells were incubated in an air shaker at 300 rpm for 16 h at 37°C and then pelleted at 11,000 rpm for 10 min at 4°C. The supernatant containing the

phage was precipitated with 0.25 volumes of 20% PEG/NaCl (20% (w/v) polyethylene glycol 8000, 2.5 M NaCl) and incubated for 30 min at 4°C. The phage were repelleted as above and resuspended in 200 µl TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). The suspension was extracted twice with 200 µl Tris-HCl-saturated phenol, pH 7.5 (United States Biochemical, USB), and chloroform (1:1), and once with 200 µl chloroform only. The single-stranded DNA was precipitated in 0.3 M sodium acetate (NaOAc) and 2.5 volumes of 95% ethanol (EtOH), pelleted at 13,000 rpm for 15 min at 4°C, and the pellet dried in a Speedvac (Savant). The pellet was resuspended in TE buffer and stored at -20°C. The yield of ss pBSSV-P/C was approximately 55 µg.

Mutagenesis was then carried out using 2 µg of the single-stranded template and 1.6 pmol of a phosphorylated mutant oligonucleotide. The protocol outlined for this system is shown in Figure 1 (RPN 1526 protocol booklet, Amersham). Briefly the mutagenic oligonucleotide was annealed to the ssDNA template and extended by T7 DNA polymerase in the presence of α-thio-deoxycytosine triphosphate (dCTPαS). NciI was used to nick the non-mutant strand while exonuclease III was used to digest it leaving only a short fragment. DNA pol I and DNA ligase were introduced to repolymerize and ligate this strand, thus generating a double-stranded DNA plasmid with the mutation of interest. The products were then transformed into $CaCl_2$

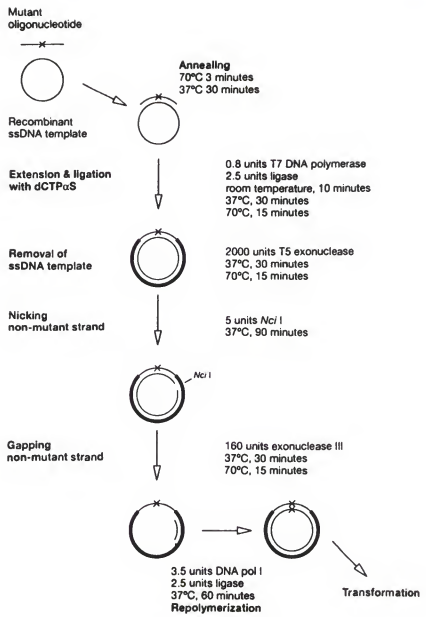


Fig. 1. Sculptor™ oligonucleotide directed *in vitro* mutagenesis protocol. RPN 1526 protocol booklet, pg. 9, Amersham.

competent E. *coli* TG1 cells and the transformation mix was plated on LB + amp plates (LB + amp, 1.5% bacto-agar) and incubated at 37°C overnight. The ampicillin-resistant colonies were screened by direct PCR amplification using the standard outside primers listed in Table 2 to generate a 688 bp DNA fragment. Briefly, a colony was picked and spotted onto a LB + amp replica plate before being added directly to a 25 µl PCR reaction mixture containing 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100, 200 µM deoxynucleoside triphosphates, 1.5 mM $MgCl_2$, 2 µM primer, and 0.5 U of Taq polymerase (Promega). 28 cycles of denaturation (50 sec, 94°C), annealing (50 sec, 40°C), and extension (1 min, 72°C) were used in addition to an initial 5 min incubation at 94°C prior to beginning the amplification cycle with the GeneAmp PCR System 9600 (Perkin Elmer). Samples of the PCR products were analyzed on a 1% (w/v) agarose (Seakem LE agarose) gel in TBE buffer (89 mM Tris-HCl, 2 mM EDTA, 89 mM boric acid) and stained by adding 0.1 µg/ml ethidium bromide to the running buffer. The DNA was then visualized on a ultraviolet (UV) illuminator box (Fotodyne, Inc.).

For the colonies which produced PCR products of the appropriate size, the remainder of the PCR reaction mixture was precipitated in 0.3 M NaOAc and 2.5 volumes 95% EtOH, pelleted at 13,000 rpm for 15 min at 4°C, and the pellet dried in a Speedvac. The pellet was resuspended and digested with the appropriate enzyme for the introduced silent

restriction site. The digests were analyzed on a 2.5% (w/v) Metaphor agarose (FMC BioProducts) gel in TBE for optimal separation of the very small digestion products. Alkaline lysis plasmid preparations were prepared for positive clones, and the mutant portions (EcoRI-NdeI) were subsequently subcloned into pGEMSV-P/C at those sites. Positive transformants were identified by PCR as above, and alkaline lysis plasmid preparations were prepared and the DNA purified by equilibrium centrifugation in continuous CsCl-ethidium bromide gradients (Maniatis *et al.*, 1989). The subcloned fragments were confirmed by sequencing.

Recombinant PCR was used to generate mutants P1, S419A, K453A, and P455/6. Site-specific mutations in the L binding site of SV P were created by introducing mismatches into the oligonucleotides used to prime the PCR amplification. This technique required two amplification steps, referred to as primary and secondary PCR, which made use of two overlapping complementary mutagenic oligonucleotide primers along with two standard outside oligonucleotide primers (Table 2). The primary PCR amplification paired each outside primer with one mutagenic primer generating a 5′-arm and a 3′-arm (Fig. 2). The "5" and "3" were designated in reference to the mutation site, where one fragment spanned the sequences upstream (5′) of the mutation through the mutation site, while the other spanned the mutation and the sequences downstream (3′) of the mutation. Therefore, two overlapping DNA fragments were

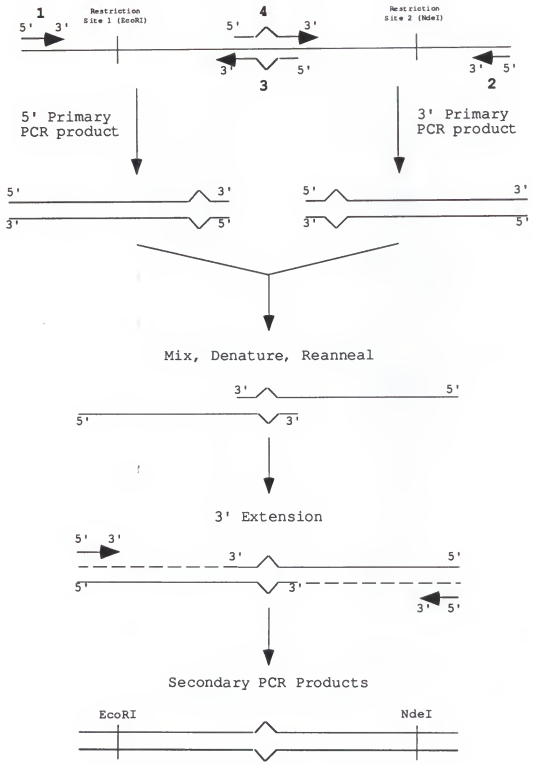


Fig. 2. Methodology for recombinant PCR site specific mutagenesis. See Materials and Methods for a description of the methodology.

generated which bore the same mutation(s) introduced via primer mismatch in the region of overlap. pGEMSV-P/C plasmid DNA was used as the template for the primary PCR amplification as described previously. The 5′ and 3′ arms were separated on a 1% (w/v) agarose TBE gel to verify size and purified from the gel using the Geneclean II kit (Bio 101, Inc.) per manufacturer's protocol. The gel purified products were mixed in varying ratios and then amplified by secondary PCR using the standard outside primers as above. The reactions were first subjected to a 5 min denaturation step at 94°C, followed by 28 cycles of denaturation, annealing and extension as described. This allowed for annealing and extension of the overlapping 5′ and 3′-arms and subsequent amplification of this fragment by the outside primers. The final full sized product was visualized on a 1% (w/v) agarose TBE gel and gel purified as before.

The purified secondary PCR product was then ligated into the pCR™II vector (TA Cloning™ Kit, Invitrogen Corporation) using T4 DNA ligase at 16°C for >16 h. The pCR™II vector allows for direct cloning of the PCR products by taking advantage of the non-template dependent activity of Taq polymerase which adds single deoxyadenosines to the 3′-end of duplex PCR products. This vector also allows for blue/white color selection as the cloning site is internal to the ß-galactosidase (*lac*Z) gene. Therefore, ligation reactions were transformed into RbCl competent *E. coli* UT481 (met- thy-

Δ(*lacPro*) r-m- *Sup* D TN10/F' *tra*D36 *pro*AB+ *lac*I^q *lacZ*ΔM15) cells and spread on LB+amp plates onto which 25 µl of 2% (w/v) X-gal had been applied. White colonies, containing an interruption in the *lac*Z gene, were picked and screened by PCR and the mutations in positive clones were verified by digestion as described previously. Positive colonies were grown and DNA prepared by alkaline lysis. From these plasmid DNAs an EcoRI-NdeI digested DNA fragment containing the mutation site was subcloned by substitution for the wild type sequence into pGEMSV-P/C at the same restriction sites. Alkaline lysis preparations were made of the PCR positive transformants and mutant plasmid DNAs purified by CsCl-ethidium bromide centrifugation. The subcloned fragments were confirmed by sequencing.

Hydrophobic-to-Alanine Site Directed Mutants

Hydrophobic-to-alanine mutagenesis targeted the putative SV L binding domain of the SV P gene from aa 408 to 473, generating 6 mutants containing 1 alanine substitution each as shown in Table 3. Recombinant PCR was used to generate mutants L421A, L425A, L428A, I430A, and G436A where the names of the mutants refer to the wild type amino acid, its position in the P polypeptide, and the amino acid substituted. Single-site mutations were created as previously described. Also as described, the putative mutants were screened by the use of a unique silent restriction site which were introduced by the mutagenic

Table 3. Hydrophobic-to-alanine mutant Sendai virus phosphoprotein genes and proteins

Mutant	Nucleotide Change[a]	Amino Acid Change[b]
L421A	C1364**G**, T1365**C**, G1366**C**	L421**A**
L425A	C1376**G**, T1377**C**, A1378**T**, T1379**A**, C1380**G**, T1381**C**	L425**A**
L428A	T1379**A**, C1380**G**, A1384**T**, C1385**G**, T1386**T**	L428**A**
I430A	T1387**G**, T1390**C**, A1391**G**, T1392**C**	I430**A**
G436A	T1408**C**, G1410**C**	G436**A**
H1	C1364**G**, T1365**C**, G1366**C**, C1376**G**, T1377**C**, A1378**T** T1379**A**, C1380**G**, T1381**C**	L421**A**, L425**A**

[a] Nucleotide changes are listed by the wt nucleotide, its position in the sequence, and the nucleotide to which it was changed in boldface characters.
[b] Amino acid changes are listed by the wt amino acid, its position in the protein, and the amino acid to which it was changed in boldface characters.

Table 4. Oligonucleotide primers for hydrophobic-to-alanine mutations

Mutant	Mutagenic primers[a]	Enzyme
L421A	SM369(+)-CAGAACTCATTGGCCATGTCCAACCTATC	NlaIII
	SM370(-)-GATAGGTTGGACATGGCCAATGAGTTCTG	
L425A	SM371R(+)-GCTGATGTCCAACGCTAGCACACTTCATATCATCAC	NheI
	SM372(-)-GTGATGATATGAAGTGTGCTAGCGTTGGACATCAGC	
L428A	SM373(+)-GATGTCCAACCTAAGTACTGCTCATATCATCACAG	ScaI
	SM374(-)-CTGTGATGATATGAGCAGTACTTAGGTTGGACATC	
I430A	SM375(+)-CAACCTATCTACACTGCACGCCATCACAGATAGAG	BsgI
	SM376(-)-CTCTATATGTGATGGCGTGCAGTGTAGATAGGTTG	
G436A	SM377(+)-CACAGATAGAGGCGCCAAGACTGACAAC	HaeII
	SM378(-)-GTTGTCAGTCTTGGCGCCTCTATCTGTG	
	Standard outside primers[a]	
	SM271(+)-ATCTACACAGGATGAGC	
	SM324(-)-GATCTCGAGCCCGGGATCTAGTTG	XhoI

[a] The oligonucleotide sequences are written 5′ to 3′ and the plus and minus symbols following the primer number refers to the messenger sense (+) or the genomic sense (-) of the oligonucleotide. The underlined sequence indicates the introduced silent restriction enzyme site shown on the right.

oligonucleotides as shown in Table 4. For all mutants, cloning of the secondary PCR products into pGEMSV-PΔEcoRI, screening for the putative mutants, plasmid DNA preparations and sequencing used the same methodology as described for the charge-to-alanine mutants.

The mutant 2S447 was created by S. Smallwood. Briefly, pGEMSV-P/C was first linearized using PpuMI which gave rise to a trinucleotide 3′ overhang of 5′ C(A/T)G 3′. The overhangs were filled in using T4 DNA polymerase and ligated using T4 DNA ligase. This resulted in a 3 bp insertion coding for a serine residue. The resulting protein thus contains two serines at aa 447 instead of one. The phosphorylation mutants S249A, S249D, and P250A were a generous gift from Dr. K. Gupta (Chicago, IL) (Byrappa et al., 1995). Serine 249 was shown to be the major phosphorylation site of the P protein and each of the above mutants was shown to be defective in phosphorylation of this residue (Byrappa *et al.*, 1995, 1996). Mutant PΔ1-325 was provided by Dr. D Kolakofsky (Geneva, Switzerland) and contains an N-terminal deletion of the P protein from aa 1-325 (Curran et al., 1991).

Infection and Transfection

As indicated in each experiment, subconfluent A549 cells in 60 mm dishes (approximately 5 x 10^6 cells) or in 35 mm dishes (approximately 1.6 x 10^6 cells) were infected with VVT7 at an m.o.i. of 2.5 pfu/cell at 37°C for 1 h in 0.5 ml or 0.2

ml, respectively, of F11 absorption media (F11 medium supplemented with 1% pen-strep, 2 mM glutamine, 14 mM HEPES, pH 7.4). At 1 h p.i. the inoculum was removed, the cells were washed with unsupplemented Opti-MEM (Gibco BRL), and 3 ml/60 mm dish or 1 ml/35mm dish of Opti-MEM supplemented as above was added to each dish. Cells were then transfected at 37°C for the times indicated in the figure legends by adding the appropriate CsCl purified plasmid(s) containing individual viral genes cloned downstream of the phage T7 promoter. Transfection mixtures used 10 µl lipofectin (Bethesda Research Laboroatories) per 2.5 µg plasmid DNA in 0.2 ml of Opti-MEM medium. The DNA-lipofection dilution was allowed to incubate at room temperature (RT) for 30 min prior to use.

Protein Analysis

Protein Synthesis *In Vivo*

In order to assess protein stability, pulse-chase analysis of the wild type and mutant proteins was performed. A549 cells in 35 mm dishes were infected with VVT7 and transfected as indicated in each experiment. At 5 h post-transfection (p.t.) the transfection medium was removed and the cells were starved in 1.5 ml Dulbecco's minimal essential medium (DMEM, Mediatech) without L-glutamine, methionine, and cysteine but supplemented with 1% pen/strep, 2 mM L-glutamine, and 14 mM HEPES, pH 7.4 for 30 min at 37°C. Proteins were then pulse-labeled by the addition of 75 µCi of

Tran^{35}S-label (ICN Pharmaceuticals, Inc.) or Expre^{35}S^{35}S trans-label (Dupont NEN) for 30 min at 37°C, and the fluid was removed. All dishes were placed at 4°C and were washed with PBS + P/S. To the chase dishes, 1 ml of medium containing 10X excess of methionine and cysteine supplemented with 1% pen/strep, 2 mM L-glutamine, 14 mM HEPES, pH 7.4, and 48 mM NaOH was added. Cells for the chase were incubated at 37°C overnight before preparation of cell extracts as described below. Total cell extracts were prepared for the pulse dishes immediately. Cells were washed with cold PBS + P/S and allowed to drain for 2 min, 100 µl of 1% (v/v) Nonidet P-40 (NP40) Lysis Buffer (150 mM NaCl, 50 mM Tris-HCL, pH 8, and 1 µg/ml aprotinin) was added and the cells incubated for 5 min. Cells were collected by scraping the dishes with a rubber policeman, vortexed to lyse the cells, and the cellular debris was removed by pelleting at 13,000 rpm for 25 min at 4°C. 50 µl of the supernatant was removed for analysis by immunoprecipitation.

For steady state protein labeling, infected and transfected cells were labeled with 100 µCi/60 mm dish of Tran^{35}S-label in DMEM without cysteine and methionine with the addition of 0.1 vol of F11 to provide 0.1X amino acids from 4 to 16 h p.t. at 37°C. Cytoplasmic cell extracts were then prepared by lysolecithin (L-α-lysophosphatidylcholine, palmitoyl; Sigma) permeabilization at 4°C. Cells were incubated on ice and washed with 3 ml/60 mm dish of wash

solution (150 mM sucrose, 30 mM HEPES, pH 7.4, 33 mM NH_4Cl, 7 mM KCl, 4.5 mM magnesium acetate). After removal of the wash solution, 1 ml/60 mm dish of lysolecithin was added to each dish and dishes rocked continuously for 1 min. The lysolecithin was diluted by promptly adding 3 ml of wash solution and the fluid aspirated. After allowing the dishes to drain for 4 min, cells were then scraped into 300 µl Sendai virus reaction mix salts (SV RM salts; 100 mM HEPES, pH 8.5, 50 mM NH_4Cl, 7 mM KCl, 4.5 mM MgOAc, 1 mM DTT, 1 mM spermidine) with or without 0.25% NP40 with a rubber policeman, and pelleted at 13,000 rpm for 25 min at 4°C. The supernatant was collected and analyzed by immunoprecipitation, bead binding, or nucleocapsid binding assays. For protein analysis by immunoblot assay, the infected and transfected cells were unlabeled and incubated for 18-22 h at 37°C while remaining in the Opti-MEM adsorption medium. Total cell extracts were prepared in 1% NP40 using the methodology indicated above.

Protein Synthesis *In Vitro*

In order to assess the ability of the mutant clones to express the desired protein, protein was synthesized *in vitro* using the TNT® Coupled Reticulocyte Lysate System (Promega) per manufacturer's protocol. Briefly, 0.5-1.0 µg of a plasmid containing the gene of choice driven by a phage T7 promoter was added to a 12 µl reaction containing rabbit reticulocyte lysate, T7 RNA polymerase, and 4 µCi ^3H-leucine.

Coupled transcription and translation was performed at 30°C for 2 h. The translation products were then separated on a mini 10% SDS-polyacrylamide gel (SDS-PAGE). The gel was fluorographed by washing 2X with DMSO for 15 min each, 1X with 22% (w/v) 2,5-diphenyl-oxazole (PPO) in DMSO (PPO/DMSO) for 30 min, and allowed to rehydrate in H_2O for 30 min. The gel was dried and the products visualized by autoradiography on Kodak X-Omat film.

Immunoprecipitation

One half of each cell extract was brought up to a final volume of 250 µl with SV RM salts with or without NP40 buffer, as indicated per experiment. The extract was preadsorbed by incubating with 100 µl of inactivated *Staphylococcus aureus* (*S. aureus;* Cowan strain, ATCC) for 30 min at 4°C. The bacteria were pelleted at 13,000 rpm for 3 min at room temperature (RT) and the supernatant transferred to a new microfuge tube. The appropriate primary antibody (1 µl) was added and incubated for 1 h at 4°C. In the case of a monoclonal antibody, the samples were incubated with secondary antibodies, both goat anti-mouse IgG and IgM (1 µl each), for 1 h at 4°C. The immunocomplexes were then incubated with *S. aureus* as above, pelleted, and washed twice with 800 µl SV RM salts with 0.25% or 1% NP40 or 800 µl RIPA [150 mM NaCl, 1% deoxycholate (w/v), 1% Triton X-100 (v/v), 10 mM Tris, pH 7.5, 0.2% aprotinin (w/v), and 0.1% SDS (w/v)] as indicated in the figure legends. The pellet was

resuspended in 25 µl of 2X lysis buffer [4% SDS, 50 mM Tris-HCl, pH 6.8, 0.2 M DTT, 40% glycerol, and 0.1% bromophenol blue (BPB)], boiled for 2 min, quenched on ice, pelleted for 3 min at 13,000 rpm and the supernatant analyzed by separation by 7.5% SDS-PAGE. Protein bands were visualized by autoradiography and quantitated on the phosphorimager (Molecular Dynamics).

Glycerol Gradient Analysis

^{35}S-labeled cytoplasmic extracts were fractionated by 5-20% (vol/vol) glycerol gradient centrifugation in the SW41 rotor at 29K for 46 hr (Horikami et al., 1992). Gradient fractions (1 ml) were collected and 0.5 ml of each was immunoprecipitated with the α-P peptide antibody. Immunoprecipitates were fractioned by SDS-PAGE. Individual viral proteins in the gel were quantitated on the phosphorimager and plotted in arbitrary units (A.U.).

Glutathione-Sepharose Bead Binding

To assess the ability of the wt or mutant SV P proteins to oligomerize, a cobinding assay was utilized which allowed complexes between P proteins and GST-P protein to be selected by binding to glutathione-coupled Sepharose beads (Glutathione Sepharose®4B, Pharmacia Biotech). A549 cells were infected with VVT7 and transfected with wt or mutant P plasmids singly or together with pGSTSV-P/C. Cell extracts were prepared in 165 µl SV RM salts + 0.25% NP40 as described. 50 µl of the cell extract was removed for

immunoprecipitation and 50 µl was brought up to a volume of 450 µl with SV RM salts. Glutathione-Sepharose beads (15 µl per reaction), preblocked in SV RM salts containing 0.1% NP-40, 0.5% nonfat dry milk (NFDM), and 10 mg/ml BSA and equilibrated in SV RM salts to a final volume of 50 µl per reaction, were added to the cell extracts and incubated for 15 min at 4°C with frequent vortexing. The beads were pelleted in a Costar centrifuge for 1 min and the supernatant was discarded. The beads were washed three times with SV RM salts + 0.25% NP40 and resuspended in a final volume of 25 µl of 2X lysis buffer, separated by 7.5% SDS-PAGE, and visualized by autoradiography.

Nucleocapsid Binding

The nucleocapsid binding assay assessed the ability of the wt or mutant polymerase complexes to bind to the viral nucleocapsid template. The wt or mutant P proteins and the wt SV L protein were co-expressed in 100 mm dishes and labeled for 1.5 h or 16 h at 5 h p.t., as indicated in the figure legends. The cells were harvested in 300 µl SV RM salts + 1 mM ATP using the lysolecithin method as previously described above. Following pelleting of the nuclei and cellular debris, cell extracts were divided into 2-125 µl aliquots to be used for binding, and a single 50 µl aliquot that was immunoprecipitated. All samples, except those for immunoprecipitation, were then incubated in the absence or presence of the wt SV polymerase-free nucleocapsid template

(wt RNA-NP) (2.5 µg) for 30 min at 30°C. The samples were then layered over step gradients containing 2.5 ml of 30% and 50% (v/v) glycerol in 10 mM HEPES, pH 8.5, and 1 mM EDTA, and pelleted at 50,000 rpm for 1.4 h at 4°C in a SW55 rotor. The pellets were resuspended in 250 µl SV RM salts + 1% NP40 and immunoprecipitated with rabbit α-SV and α-SV-L antibodies and analyzed by 7.5% SDS-PAGE and autoradiography.

Immunoblot Analysis

Cell extracts (10 µl/100 µl) were combined with an equal volume of 2X lysis buffer, denatured by boiling for 2 min, quenched on ice, and separated by 7.5% SDS-PAGE. The gel and an equal sized piece of nitrocellulose (Schleicher and Schuell) were equilibrated by shaking in 200 ml of transfer buffer [190 mM glycine, 25 mM Tris, and 20% (v/v) methanol] for 20 min at RT. Proteins were electroblotted onto the nitrocellulose at 40 V at 4°C for 16 h using the mini-PROTEAN electrophoresis system (BioRad). The nitrocellulose was removed and incubated in blocking buffer [10% newborn calf serum (Gibco BRL), 5% bovine serum albumin (BSA) in TBS + Tween 20 (0.02 M Tris, pH 7.5, 0.5 M NaCl, 0.05% (v/v) Tween 20)] for 1 h at 45°C, followed by washing 2X with TBS + Tween 20 for 5 min each. For identification of proteins, the nitrocellulose was incubated with the appropriate primary antibody for 1 h at RT followed by a 1 h incubation with alkaline phosphatase conjugated goat anti-rabbit IgG antibody (1:2000 dilution in TBS). The nitrocellulose was washed 2X

with TBS + Tween before and after each antibody incubation. For visualization of the antibody-proteins complexes, the nitrocellulose was incubated in the presence of a solution containing nBT and BCIP (220 µl nitroblue tetrazolium, 50 mg/ml in 70% dimethyl formamide, 110 µl 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt, 50 mg/ml in 100% dimethyl formamide) in 20 ml of alkaline phosphatase buffer (100 mM Tris, pH 9.0, 100 mM NaCl, 5 mM $MgCl_2$) with rocking, and the reaction was stopped by the addition of water upon color development. For identification of proteins using the Enhanced Chemiluminescence (ECL) protein identification system (Amersham Life Science), the secondary antibody utilized was horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG and washes were done in PBS. Complexes were visualized as outlined in the manufacturer's protocol.

RNA Synthesis

In Vitro RNA Transcription

Wt or mutant SV P plasmids and wt SV L were cotransfected into VVT7-infected cells in a 60 mm dish with 1.5 µg and 0.5 µg, respectively, along with a negative control containing no plasmid DNA. At 18 h p.t., lysolecithin cytoplasmic extracts were prepared as above in 115 µl of SV RM salts supplemented with 1 mM each of ATP, GTP, and UTP, 10 µM CTP and 10% glycerol (SV RM incomplete). The cells were lysed by pipetting up and down 15X, the nuclei pelleted at 4°C for 5 min at 1,600 rpm, and the supernatant

divided into aliquots of 10 µl, to be used in immunoblot analysis, and 90 µl, to be used for *in vitro* transcription. For digestion of endogenous RNA and plasmids present in the extracts, each sample was incubated with 20 µg/ml micrococcal nuclease (MN) in the presence of 1 mM $CaCl_2$ at 30° for 30 min followed by inactivation with 7.5 mM EGTA (ethylene glycol-bis(*B*-aminoethyl ether)-*N,N*-tetraacetic acid, pH 8.0).

For RNA transcription, the nuclease treated extracts were then supplemented with 0.1 volume of a 10X supplemental mix [45 mM MgOAc, 5 U/µl RNasin, 200 µg/ml actinomycin D, 400 U/ml creatine phosphokinase (CPK), and 33 mg/ml creatine phosphate], 1 µg of polymerase-free wt SV RNA-NP template, and 20 µCi of [α-^{32}P]CTP (3000 Ci/mmol, Amersham, Dupont). Transcripts were synthesized at 30°C for 2 h. The transcription products were purified by pelleting through 5 ml CsCl step gradients prepared by layering from the bottom 1 ml 5.7 M CsCl in 50 mM EDTA, pH 7.5, cushion, 1.5 ml of 40% (w/w) CsCl in TNE (25 mM Tris-HCl, pH 7.5, 50 mM NaCl, 2 mM EDTA), 1.5 ml 20% (w/w) CsCl in TNE, and 0.85 ml 30% (v/v) glycerol in SV RM salts in an SW55 rotor at 36,000 rpm for 16 h at 4°C. The pelleted transcripts were resuspended in 270 µl of low salt buffer (LSB, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA) and precipitated with 0.3 M NaOAc, pH 5.6 and 2.5 volumes 95% EtOH. Alternatively, total RNA was isolated through the use of the Quiagen RNeasy™ Total RNA Kit per manufacturer's protocol and ethanol precipitated. The total

RNA products were pelleted at 13,000 rpm for 15 min at 4°C and dried in a speedvac. Pellets were resuspended in 15 µl of sample buffer (2.5 mM citrate buffer, pH 3.5, 6 M urea, 20% sucrose, 5 mM EDTA, 0.012% BPB), denatured by boiling for 2 min, and quenched on ice. The RNA was analyzed directly by electrophoresis on a 10 cm horizontal 1.5% agarose-6 M urea gel. The gel was dried on thick filter paper for 3 h at 72°C (Biorad, Gel Dryer Model 543), exposed to film at -70°C with an intensifying screen, and the products quantitated on a phosphorimager.

For the transcription rescue experiments, 60 mm dishes of VVT7-infected cells were transfected with no plasmids or wt or mutant SV P plasmids alone (5 µg) and separate dishes cotransfected in duplicate with the same P plasmids and wt L plasmid at a 1:1 ratio (3 µg each). It has been shown previously that this ratio of P to L is suboptimal for *in vitro* transcription (~25%). In an effort to maintain the same concentration of viral polymerase as for the *in vitro* transcription experiments, the amount of P plasmid was doubled from 1.5 to 3 µg since only 60% of the extract was used for the reaction as will be discussed. Cell extracts were prepared in 110 µl of SV RM salts (incomplete), duplicate dishes pooled, and nuclei pelleted as before. Cell extracts containing the wt or mutant viral polymerases were then divided into three 60 µl aliquots and to these were added 30 µl of a cell extract which contained either mock, wt

P, or mutant P protein, where the P proteins were expressed alone. *In vitro* transcription was performed and analyzed as described.

Leader RNA Synthesis

Leader RNA synthesis from SV wt RNA-NP template was analyzed by Northern analysis. Leader RNA was synthesized and processed as described for *in vitro* transcription except that the CTP concentration was increased to 1 mM and the reaction was allowed to proceed in the absence of radioactive nucleotides. The cytolasmic extract was treated with proteinase K, extracted with phenol/CCl_3, and the ethanol precipitated product RNAs were pelleted in a microfuge at 13,000 rpm for 15 min at 4°C and dried in a Speedvac. The RNA pellet was resuspended in 15 µl of H_2O and an equal volume of Stop Mix (94% formamide, 0.3 mg/ml xylene cyanol, 0.3 mg/ml BPB, 12.5 mM EDTA), denatured, and separated on a pre-run (250-300 V for 45 min) 8% polyacrylamide-8 M urea gel at 300-400 V until the BPB had run 2/3 of the way through the gel. The gel was washed 2 times at 20 min intervals in Transfer Buffer and the RNA was electroblotted onto Hybond-N nitrocellulose (Amersham) at 35-40 V at 4°C for 19-24 h using the Trans-Blot Cell system (BioRad). The RNA was UV crosslinked (Stratalinker UV Crosslinker 1800, Stratagene) to the nitrocellulose which was kept wet by first placing it on filter paper which was premoistened with 5X SSC (0.75 M NaCl, 75 mM sodium citrate).

The blot was incubated for at least 2 h at 45°C in 20 ml of boiled and quenched prehybridization buffer [50% formamide, 4X Denhardts, 0.05% SDS, 2.5X SSPE (0.9 M NaCl, 2.5 N NaOH, 5 mM EDTA, 50 mM sodium phosphate, pH 7.4), 200 µg/ml salmon sperm DNA] in a plastic bag. A ^{32}P-CTP labeled *l*-NP riboprobe (see Probes) synthesized according to manufacturer's protocol was added to the prehybridization solution and incubated 18 h at 45°C. The blot was washed 2 times in 5 min intervals in 6X SSPE/0.1% SDS at room temperature, followed by 1 wash in 6X SSPE/0.1% SDS at 55°C for 30 min, 1 wash in 2X SSPE/1% SDS at 55°C for 30 min, and a final wash in 0.1X SSPE for 30 min at RT. The blot was then exposed to film and the RNA product analyzed on the phosphorimager.

CHAPTER 3
FUNCTION OF P PROTEIN PHOSPHORYLATION IN VIRAL RNA SYNTHESIS

Introduction

The phosphorylated P protein is a required subunit of the viral RNA dependent RNA polymerase. Based on recent evidence for several viruses, it has been proposed that the phosphorylation state of P may play a role in the regulation of polymerase activity. Regulation may occur by influencing the pattern of RNA synthesis directed by the polymerase or via interactions of the P subunit with other viral or yet undefined cellular proteins. For instance serine 60 (S60) and threonine 62 (T62) of the VSV P protein are constitutively phosphorylated by cellular casein kinase II (CKII), and both phosphorylations are required for P protein oligomerization (Barik and Banerjee, 1991, 1992; Beckes and Perrault, 1992; Gao and Lenard, 1995a, b; Das et al., 1995). The oligomerized P proved to be the transcriptionally active form of the protein and was necessary for both complex formation between the P and the L subunits of the polymerase, as well as assembly of the active polymerase onto the nucleocapsid template via binding through P protein (Gao and Lenard, 1995a,b). Similar studies on the RSV P protein showed that S232 and S237 are the major sites of

phosphorylation and that phosphorylation is also directed by cellular casein kinase II (Mazumder et al., 1994a, b; Barik et al., 1995). Mutational analyses of these residues also have demonstrated that phosphorylation at these sites is necessary for the transcriptional activity of the polymerase.

Studies by Byrappa et al. (1995, 1996) have identified the prominent phosphorylation site in the SV P protein as S249. Since mutation of P250 to alanine also caused a defect in phosphorylation at S249, and there is no consensus sequence at this site for CKII, it has been proposed that phosphorylation is mediated by a proline-directed serine kinase. Three mutants defective in phosphorylation, S249A, S249D, and P250A, were generously provided by Dr. S. Byrappa and Dr. K. Gupta (Chicago, Il). The strategy behind these changes is as follows: First, mutagenesis of a charged residue to alanine is based upon the belief that the change to a non-polar, non-aliphatic amino acid may disrupt function while maintaining the overall structure of the protein. Second, by changing a serine to an aspartic acid, it may be possible to substitute the phosphate group with a negatively charged side chain thereby maintaining function. To elucidate the function of P protein phosphorylation for this member of the paramyxovirus family, two approaches were chosen. The first was to assess the ability of the mutant P proteins to form the protein-protein interactions necessary for RNA synthesis by testing for P-P, P-L, and polymerase-

RNA-NP (P/L-NC) complexes. The second set of experiments focused on the biological activity of the mutant P proteins by testing for viral transcription *in vitro*.

Results

P Phosphorylation Mutants are Stably Expressed

In order to determine if the P phosphorylation mutants were stably expressed in cell culture, a pulse-chase experiment was performed as described in Materials and Methods. A549 cells infected with VVT7 were transfected in duplicate with each of the wt or mutant P plasmids alone and at 5 h post-transfection (pt) the cells were labeled with Tran^{35}S-label for 30 min. The pulse dishes were harvested immediately, and the second set of dishes were chased overnight and cytoplasmic cell extracts prepared. The viral proteins were identified by immunoprecipitation using an α-P peptide antibody and analyzed by SDS-PAGE. In the absence of transfected plasmid, some vaccinia virus proteins were shown to be non-specifically immunoprecipitated (Fig. 3, lanes 1 and 2). The wt P protein (~70 KDa) was expressed in the pulse (Fig. 3, lane 3) and a significant fraction was stable to the chase (~30%) (lane 4). The chase band appears to migrate at a slightly larger molecular weight consistent with the idea that the P protein is modified by phosphorylation, which may be incomplete within the first 30 min of synthesis. Each of the phosphorylation mutants was similarly synthesized with a portion stable to the chase (Fig. 3, lanes 5-10).

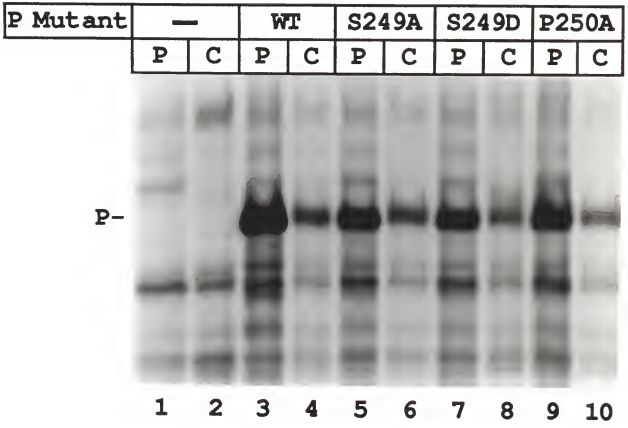


Fig. 3. Pulse-chase analysis of the wt and phosphorylation mutant P proteins. A549 cells infected with VVT7 were transfected in duplicate with no plasmids (-), or the wt (WT) or mutant P plasmids (1.7 μg) as indicated at the top. At 5 h pt the cells were pulse labeled with Tran^{35}S-label and extracts prepared immediately (pulse, P) or at 16 h pt following a chase (C) as described in Materials and Methods. The viral proteins were immunoprecipitated with α-P peptide antibody and analyzed by 7.5% SDS-PAGE. The position of the P protein is indicated.

Although these mutants are defective in phosphorylation at the primary phosphorylation site, the minor sites are still capable of being phosphorylated (Byrappa et al., 1995, 1996) leading to the shift in the apparent molecular weight (Fig. 3, lanes 6, 8, and 10). These data show that each of the P phosphorylation mutants is as stable as wild type during expression in cells.

The Mutant P Proteins are Capable of Oligomerization

To ascertain whether or not the P phosphorylation mutants were capable of oligomerization, the wt or mutant P proteins were tested for binding to the GST-P fusion protein, where cobinding of P with GST-P to glutathione-Sepharose beads is a measure of complex formation. No plasmids (-) or wt or mutant P plasmids were transfected alone or together with GST-P plasmid into VVT7 infected A549 cells. The cells were labeled with Tran^{35}S-label and samples of cytoplasmic cell extracts were immunoprecipitated or incubated with glutathione-Sepharose beads and analyzed by SDS-PAGE. In the mock transfected samples there were some vaccinia background bands (Figs. 4A and B, lanes 1). When the wt P protein was expressed alone, it did not bind to the beads (Fig. 4B, lane 2) even though the P protein was expressed (Fig. 4A, lane 2). However, when the P protein was expressed in the presence of GST-P, the P protein cobound with GST-P to the beads (Fig. 4A and B, lanes 3) showing complex formation. The band which migrated directly above the wt P protein has been shown to be

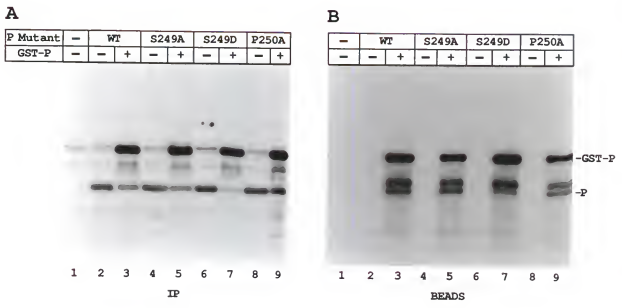


Fig. 4. Cobinding of the phosphorylation defective mutant P proteins and the GST-P fusion protein to glutathione-Sepharose beads. A549 cells infected with VVT7 were transfected with no plasmids (-), wt P plasmid (WT) or mutant P plasmids (1.7 μg) alone or together with GST-P plasmid (0.17 μg) as indicated. At 5 h pt the cells were labeled for 2 h with Tran^{35}S-label and extracts prepared as described in Materials and Methods. Samples were A) immunoprecipitated (IP) with the α-P peptide antibody or B) incubated with glutathione-Sepharose beads (Beads) and the proteins analyzed by 7.5% SDS-PAGE. The positions of the P and GST-P proteins are indicated.

a breakdown product of the fusion protein which still contained the GST moiety and was capable of binding to the beads (data not shown). Each of the P mutants also cobound to beads when expressed in the presence of the GST-P protein (Figs. 4A and B, lanes 5, 7, and 9), but not when expressed alone (Figs. 4A and B, lanes 4, 6, and 8). It was previously shown that the P protein does not bind to GST, and therefore, the interaction is due specifically to the P portion of the fusion protein (Chandrika et al., 1995). These data suggest that the S249A, S249D and P250A mutant proteins are still capable of oligomerization. The mutants were then tested for their ability to form a stable polymerase complex capable of assembly onto the nucleocapsid template.

Mutant P Proteins Assemble onto the Nucleocapsid Template

Previous work has shown that the P protein is able to bind to the RNA-NP (NC) either alone or within the P-L complex. Thus P binding assembles the P-L complex on the template to initiate RNA synthesis (Portner and Murti, 1986; Portner et al., 1988; Ryan et al. 1988, 1990, 1991; Morgan, 1991; Horikami and Moyer, 1995). To test the ability of the mutant P proteins within the polymerase complex to bind to the template, an assay was employed where bound proteins are pelleted with nucleocapsids. A549 cells were VVT7 infected and transfected in duplicate with no plasmids (-) or with wt or mutant P plasmids together with L plasmid. The cells were radiolabeled with ^{35}S amino acids and cytoplasmic cell

extracts were prepared. Twenty percent of each sample was used for immunoprecipitation to determine the total viral protein synthesized, while the remainder was divided and incubated in the absence or presence of NC. The nucleocapsids and associated proteins were pelleted, immunoprecipitated, and analyzed by SDS-PAGE. In the absence of transfected plasmid, some vaccinia virus proteins were non-specifically immunoprecipitated and/or pelleted with the antisera (Fig. 5A, lane 1; and Fig. 5B, lanes 1 and 2, respectively). When the wt or mutant P and L proteins were coexpressed and pelleted in the absence of NC, no P was present in the pellet but a faint band of the L protein was visualized (Fig. 5B, lanes 3, 5, 7, and 9). In the presence of NC both the WT and mutant P and significantly more L protein were in the pellets, and thus all polymerases bound nucleocapsids (Fig. 5B, lanes 4, 6, 8, and 10). Although there appears to be some differences between the amount of pelleting and thus, binding between the various mutants, this may be explained by the differential in protein expression shown by immunoprecipitation of the total protein. It should be noted that expression of S249D relative to the wt and mutant P proteins was somewhat decreased in each of the protein experiments (Fig. 3-5). These data present evidence that the mutant P proteins are capable not only of binding to the nucleocapsid template, but of assembling the polymerase complex onto the template as well. Since the P mutants

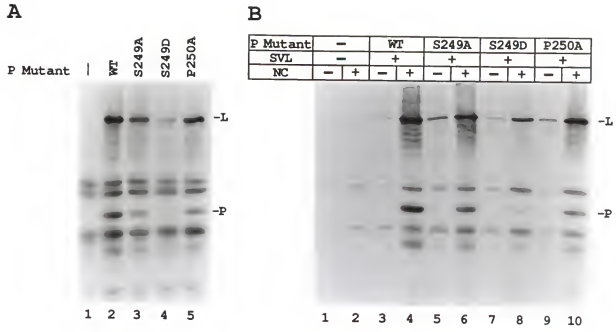


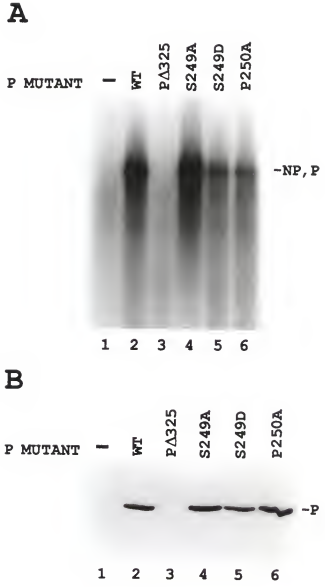
Fig. 5. Binding of the wt and phosphorylation defective mutant polymerase complexes to the wt nucleocapsid template. A549 cells infected with VVT7 were transfected with no plasmids (-) or cotransfected in duplicate with wt P (WT) or mutant P plasmids (5 μg) and wt L plasmid (5 μg) as indicated. Cells were radiolabeled for 2 h with Tran^{35}S-label, cytoplasmic cell extracts were prepared, and duplicated dishes pooled as described in Materials and Methods. A) 20% of the sample was immunoprecipitated with the α-P peptide and α-L antibodies to determine total protein synthesized and analyzed by 7.5% SDS-PAGE. B) The remaining extract was divided, incubated in the absence or presence of polymerase-free wt RNA-NP (2.5 μg, NC), and pelleted through glycerol. The pellets were collected and analyzed by 7.5% SDS-PAGE. The positions of the P and L proteins are indicated.

proved to form stable interactions in each of the protein-protein complexes necessary for viral RNA synthesis, the mutants were next tested for biological activity.

The P Mutants are Active in Transcription *In Vitro*

In order to ascertain whether the amino acid changes at the primary phosphorylation site of the P protein affected polymerase function, the mutant P proteins were tested for their ability to transcribe mRNA from a wt RNA-NP template. VVT7 infected A549 cells were cotransfected with wt or mutant P plasmids together with L plasmid at concentrations optimal for *in vitro* transcription (Horikami et al., 1992). Cytoplasmic extracts were prepared, incubated with polymerase-free wt RNA-NP and the total mRNA products analyzed as described in Materials and Methods. The VVT7 infected, but non-transfected negative control yielded no transcription products (Fig. 6A, lane 1) showing there was no endogenous polymerase activity associated with the template. The wt P and L proteins were active in mRNA transcription (Fig. 6A, lane 2). The major band contains both the NP and P mRNAs which comigrate on this gel. The mutant protein PΔ325 which does not contain the first 325 aa of the P ORF and therefore, does not include the primary phosphorylation site, was inactive (Fig. 6A, lane 3) as reported earlier (Curran et al., 1991). The mutant P protein S249A was active in transcription *in vitro* at levels comparable to wt P (Fig. 6A, lane 4) as quantitated on the phosphorimager from three

Fig. 6. *In vitro* transcription of the SV wt RNA-NP with the P proteins S249A, S249D, and P250A. A) A549 cells were VVT7 infected and transfected with no plasmids (-) or cotransfected with wt or mutant P plasmids (1.5 μg) together with L plasmid (0.5 μg). Cytoplasmic cell extracts were prepared at 18 h pt and incubated with SV wt RNA-NP in the presence of [α-^{32}P]CTP. The transcripts were purified on Quiagen RNeasy™columns per manufacturer's protocol and the purified RNA analyzed on a 1.5% agarose-6 M urea gel. The relative positions of the NP and P transcripts are indicated. B) Immunoblot analysis using enhanced chemiluminescence on samples (10%) of the cytoplasmic extracts used for *in vitro* transcription with an α-P peptide antibody as described in Materials and Methods. The position of the P protein is indicated.


A
P MUTANT
–
WT
PΔ325
S249A
S249D
P250A
-NP, P
1 2 3 4 5 6
B
P MUTANT
–
WT
PΔ325
S249A
S249D
P250A
-P
1 2 3 4 5 6

separate experiments (120%). However, the mutant P proteins S249D and P250A were on average only 28% and 34% as active as wt P, respectively (Fig. 6A, lanes 5 and 6). The wt and phosphorylation defective P mutants were nearly equally expressed in these extracts as shown by immunoblot analysis of the extracts as described in Materials and Methods (Fig. 6B, lanes 2 and 4-6). The mutant PΔ325 is not detected on this blot, but it was previously determined to be expressed to the same degree as each of the other P proteins (data not shown). Thus, these data suggest that phosphorylation at S249 is not required for polymerase activity.

Discussion

To elucidate the role of P protein phosphorylation in Sendai virus RNA synthesis, three proteins defective in phosphorylation by mutation at or adjacent to the major phosphorylation site, S249, were studied. We first assessed the ability of the mutant proteins to form the protein-protein interactions required for viral RNA synthesis. To achieve full transcriptional activity, the P protein must complex with the L protein to form the active RNA-dependent RNA polymerase and assemble this complex onto the template. The data presented here provide evidence that the SV phosphorylation defective mutants, S249A, S249D, and P250A, are all competent for each of these protein-protein interactions necessary to achieve biological activity including P oligomerization (Figs. 4 and 5). Recent evidence

has shown that oligomerization of the VSV P protein is required for binding to L and assembly onto the nucleocapsid (Gao and Lenard, 1995). However, deletion analysis revealed that the SV P protein is still able to form the P-L complex when the P oligomerization domain is absent, although the biological activity of this complex was not tested (Smallwood et al., 1994; Curran et al., 1994). This is in contrast to the VSV data and points to possible differences in the formation of the polymerase complex between these members of the paramyxovirus and rhabdovirus families.

We next tested for biological activity directly. To look specifically at polymerase function, *in vitro* transcription was carried out with each of the mutant P proteins (Fig. 6). While mutant S249A retained wt activity, mutants S249D and P250A gave reduced synthesis of viral mRNAs. To fully assess the importance of phosphorylation at S249, replication of a DI-H template must also be assayed. Replication requires not only the active polymerase complex, but also a soluble $P\text{-}NP_0$ complex that serves to encapsidate the nascent viral RNA during replication. If the mutants are defective in DI-H replication, one could postulate that the phosphorylation site is necessary for either encapsidation or encapsidation substrate formation, but not for polymerase activity. To determine if phosphorylation is important for encapsidation during replication, each mutant was assayed for the ability to replicate virion RNA *in vitro*. Preliminary

evidence indicates that the mutant P proteins' replicative activities are similar to their transcriptional activities (data not shown). Although these results must be confirmed, these data reveal that phosphorylation is not required for encapsidation. In summary, these data provide evidence that the phosphorylation of the P protein at S249 is unnecessary for RNA synthesis *in vitro*.

The reduction in activity of S249D and P250A may be due to aberrant folding of these proteins. Serine residues have a hydroxymethyl side chain while alanine residues have a methyl group only. Thus, mutation from a serine to an alanine may not significantly affect protein structure. Mutation to an aspartic acid, however, necessitates the acceptance of a bulky and negatively charged carboxyl group in place of the hydroxyl group which may alter protein structure and/or stability more significantly. It may be argued that since S249 is phosphorylated, the attached phosphate group on the serine would act in a similar fashion to a carboxyl group. It may also be true, though, that proper folding of the protein must occur before phosphorylation can result. In this instance a serine to aspartic acid mutation may not be fully functional. The proline to alanine substitution may also prove to be detrimental to the folding process. Proline is an imino acid and its five-membered ring dictates a rigid structure. Proline is generally found in β-turns and has been shown to

disrupt α-helices. Thus mutation of a proline to any other amino acid could damage protein structure depending on its importance at any given position. Because each mutant retained all of the wt protein-protein interactions (Fig. 4 and 5), we propose that S249D and P250A gave decreased activity in *in vitro* transcription due to a mild alteration in protein structure. Since each protein proved stable to an overnight chase (Fig. 3), we do not believe the stability of these mutant proteins is of primary importance, although such a problem cannot be ruled out with the S249D protein.

Another point of interest should be noted for the purpose of studying the effects of phosphorylation in protein function. Studies by Chattopadhyay et al. (1987) showed that substitution of either S236 or S242 of the P protein of VSV New Jersey strain to alanine prevented phosphorylation at these sites with a concomitant loss of transcriptional activity. Thus, it was implied that phosphorylation of these residues was critical for P function. Likewise, Gao and Lenard (1995) determined that similar mutations of VSV Indiana strain P protein of S227 and S233 to alanine disrupted template binding and transcriptional activity of the viral polymerase. It was also shown that mutation of these residues to aspartic acid had no effect on transcription. Thus, it initially appeared that phosphorylation was necessary for P activity and that the aspartate residues substituted for the phosphorylated

serines. However, further investigation revealed that the unphosphorylated S227 and S233 were as active as wild type in transcription. Thus, serine, serine phosphate, and aspartic acid were equally acceptable at these positions whereas alanines were not and thus, phosphorylation was irrelevant. Therefore, phosphorylation studies utilizing mutational analyses should be used with caution.

Future Plans

These studies show that the phosphorylation defective mutants retained wt P protein function which was unexpected. Studies of other paramyxovirus and rhabdovirus P proteins have demonstrated that the major phosphorylation site is absolutely necessary for P function in RNA synthesis. It is important to note, however, that the *in vitro* replication assay uses an excess of input nucleocapsids and is thus believed to measure only a single round of RNA synthesis and encapsidation (Curran et al., 1993). Therefore, it cannot be ruled out that the newly encapsidated RNA cannot function properly as a replicative template. To address this, we will perform *in vivo* analyses using a T7 driven plasmid containing the cDNA of the DI-H genome. When cotransfected into cells with the plasmids containing the genes for the P, L and NP proteins, the DI-H RNA is encapsidated by the expressed NP protein and may then serve as a replicative template. This methodology measures amplification of the DI-H genome and may

be used as a measure of template function (Curran et al., 1993).

Also, in an effort to determine any possible role of phosphorylation, future studies will be focused on the V protein. The V protein is made from an edited P mRNA whereby the viral polymerase adds a single guanosine at nt 1053, presumably by a stuttering mechanism similar to polyadenylation, and this causes a -1 frameshift of the P ORF during the translation of the edited mRNA (Vidal et al., 1990a, b; Pelet et al., 1991; Kolakofsky et al., 1991). The resultant V protein thus contains a unique cysteine rich carboxy-terminal end, but is amino-coterminal with the P protein from aa 1-317 and thus contains S249 as shown in Fig. 7. Previous studies found that V was phosphorylated to nearly the same extent as P during the natural viral infection (Curran et al., 1991). The V protein has been shown to have an inhibitory effect on viral replication both *in vivo* and *in vitro* (Curran et al., 1991).

Recently our lab has provided evidence that the V protein interacts directly with the NP_0 protein (Horikami et al., 1996) suggesting that this interaction is responsible for the inhibitory effect seen with V on nucleocapsid replication. Deletion studies have suggested that two regions, aa 33-41 and 78-317 of V are responsible for the V-NP_0 interaction (Curran et al., 1995, Horikami et al., 1996).

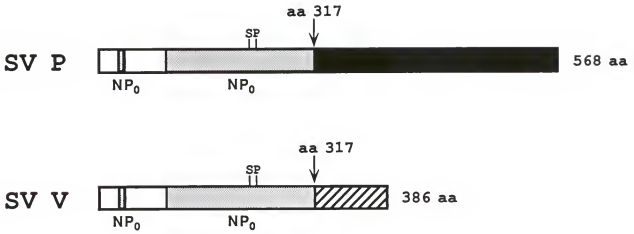


Fig. 7. A schematic comparison of the Sendai virus P and V proteins. The arrow indicates the site of the frameshift. The black and striped boxes indicate the unique carboxy-terminus of the P and V proteins, respectively. The stipled boxes represent interaction domains for each protein with NP_0. The SP refers to the location of the major phosphorylation site, S249 and P250, of the P protein.

The second region contains the major phosphorylation site S249 and makes V a good candidate for similar phosphorylation studies as were performed for P. Each of the phosphorylation mutations will be cloned so that they will contain the S249 and P250 mutations in the context of the V protein. To determine, then, if phosphorylation is necessary for V function, analysis of the V-NP_0 protein-protein interaction as well as the inhibitory effect of V on replication both *in vivo* and *in vitro* will be tested. Since the *in vivo* replication assay assesses amplification of replication products, it may prove to be a more sensitive assay for inhibition of genome synthesis. It remains to be determined, therefore, if the phosphorylation site, S249, is required for V protein function.

CHAPTER 4
IDENTIFICATION OF CHARGED AMINO ACIDS IMPORTANT FOR L BINDING

Introduction

The Sendai virus RNA-dependent RNA polymerase is composed of two subunits, the L protein and the P protein. The L protein is proposed to contain all of the catalytic activity of the polymerase complex, while a function for the P subunit has yet to be defined. Through deletion analysis, the binding domain for L within the P protein has been assigned to aa 412-479 (Smallwood et al., 1994; Curran et al., 1994). It was our primary goal to characterize the P-L interaction with respect to the specific amino acids which are involved within the P protein. In order to accomplish this we chose two different strategies. The first strategy involved the construction of chimeric proteins which would contain only the L binding domain of the P protein. This would enable us to determine whether this domain was necessary and sufficient to form a complex with the L protein. Curran et al. (1995) showed that such an approach for a different protein was feasible. They presented data whereby aa 291-380 of MV P protein, a putative P-P binding site, fused to the C-terminus of the monomeric *Xenopus laevis* La protein promoted oligomerization of the fusion protein.

We have constructed two chimeric proteins between the SV P and the MV P proteins in which the SV L and the MV P/L binding domains (Bankamp et al., submitted) were swapped into the heterologous protein fused to GST as shown in Fig. 8. We will show evidence that these domains alone are incapable of interacting with their homologous partners in complexes.

The second strategy involved direct mutation of amino acids within the L binding domain. We selected clustered charge-to-alanine mutagenesis as our approach to this problem because of the likelihood of targeting residues residing on the surface of the protein and producing stable mutants capable of exhibiting altered phenotypes (Cunningham and Wells, 1989; Bass et al., 1991; Bennett et al., 1991; Wertman et al., 1992). This approach was initially used to target protein interactions between the human growth hormone and its receptor (Cunningham and Wells, 1989). This study showed that a majority of the mutants (81%) were stably expressed with 24% having altered binding affinities. Clustered charge-to-alanine mutagenesis has since been used for the identification of surface residues involved in protein-protein interactions (Bass et al., 1991; Bennett et al., 1991), catalytic activities (Diamond and Kirkegaard, 1994), and for the creation of temperature sensitive mutants (Wertman et al., 1992; Hasset and Condit, 1994; Diamond and Kirkegaard, 1994). Using this method we targeted 19 aa of the P protein from aa 408 to 473 for mutagenesis as given in

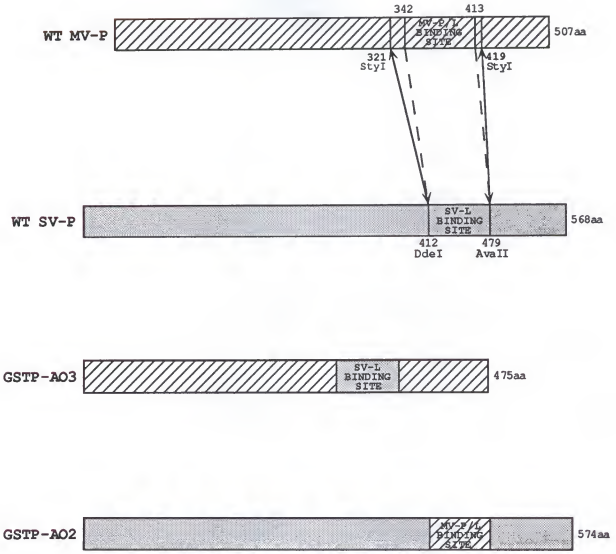


Fig. 8. Sendai and measles virus P protein chimeras. For a description see Materials and Methods.

Table 1 of Materials and Methods. These amino acids were chosen due to their charge as well as their conservation among the parainfluenza- and morbilliviruses (Fig. 9). The mutations were changed singly or in groups so as to span the entire region as represented in Fig. 10. We have also changed two completely conserved serines to alanines (Fig. 9 and 10). We will present evidence that all of these mutants maintain their ability to complex with L protein, but some proved defective in RNA synthesis.

Results

The Chimera GSTP-AO3 is Unable to Bind SV L

One chimera, GSTP-AO3, that was prepared substituted the SV L binding site from SV P into a somewhat similar site in the MV P protein (Fig. 8). To ascertain whether or not the chimeric protein GSTP-AO3 contained a functional L binding site, the L protein was tested for its ability to bind to the chimera, where cobinding of L with the GST fusion protein to glutathione-Sepharose beads is a measure of complex formation. Chandrika and coworkers (1995) showed that the GST-SVP fusion protein retains the ability to bind L. No plasmids (-) or GSTP-AO3 and L plasmids were transfected alone or together into VVT7 infected A549 cells. When the GSTP-AO3 protein was expressed alone, it bound to the beads (Fig. 11B, lane 2). Its position is indicated by the asterisk. When wt L protein was expressed alone, it did not bind to the beads (Fig. 11B, lane 3) even though the L

```
          aa408
                          .               .               .
SV-P      KRFSEYQK EQNSLLMSNL STLHIITDRG GKTDNTDSLT
hPIV3-P   RRLIENQR EQLSLITSLI SNLKIMTERG GKKDQNESNE
bPIV3-P   RRLIENQK EQLSLITSLI SNLKIMTERG GKKDQPENSG
MV-P      ............STLEGHL SSIMIAIPGL GKDPNDPTAD
RV-P      ............STIEGHL SSFMIAIPGF GKDPNDPTAD
CDV-P     ............STIEGHL SSIMIAIPGF GKDTGDPTAN

                                                    aa479
                                        .
SV-P      RSPSVFAKSK ENKTKATRFD PSMETLEDMK .YKP..D
hPIV3-P   RVSMIKTKLK EEKIKKTRFD PLMEAQGIDK .NIP..D
bPIV3-P   RTPMIKTKAK EEKIKKVRFD PLMETQGIEK .NIP..D
MV-P      VEINPDLKPI IGRDSGRAL. ..AEVLKKPV ASRQLQG
RV-P      VDINPDLRPI IGRDSGRAL. ..AEVLKKPA SERQSKD
CDV-P     VDINPELRPI IGRDSGRPL. ..AEVLKQPA SSRGNRK
```

Fig. 9. A) Sequence comparison between the parainfluenza- and morbillivirus P proteins from amino acids (aa) 408 to 479. The bold characters refer to the charge-to-alanine P mutants and two conserved serine residues. Amino acids conserved throughout both genuses are indicated by a dot above the column. (SV, Sendai virus; hPIV3, human parainfluenza virus type 3; bPIV3, bovine parainfluenza virus type 3; MV, measles virus; RV, rinderpest virus; CDV, canine distemper virus)

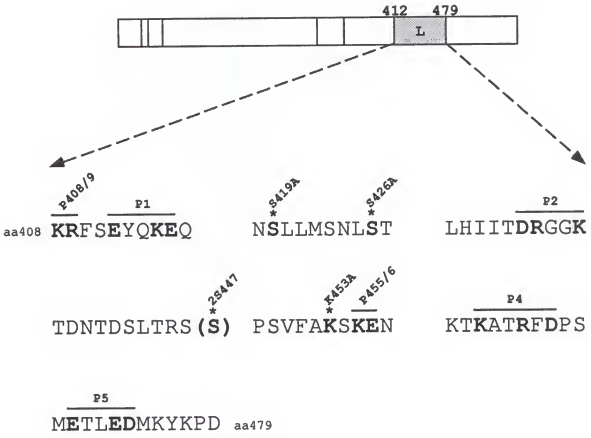


Fig. 10. A schematic of the P protein and the aa sequences from 408 to 479 of the charge-to-alanine P mutants. Alanine was substituted for one to three charged residues in the mutant P proteins and these residues are highlighted by bold lettering. Single changes are indicated by an asterisk while grouped changes are indicated by an overline.

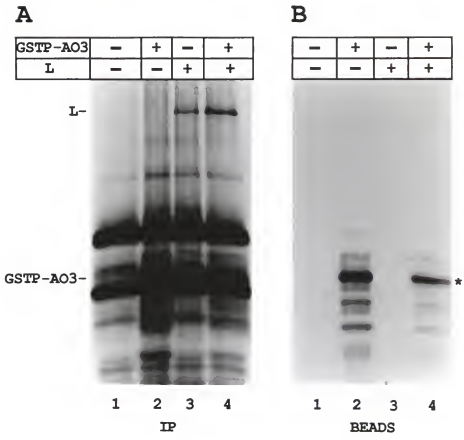


Fig. 11. Cobinding of the SV-L protein and the GSTP-AO3 fusion protein to glutathione-Sepharose beads. A549 cells infected with VVT7 were transfected with no plasmids (-), wt P plasmid (WT) or mutant P plasmids (1.7 μg) alone or together with GST-P plasmid (0.17 μg) as indicated. AT 5 h pt the cells were labeled for 5 h with Tran^{35}S-label and extracts prepared as described in Materials and Methods. Samples were A) Immunoprecipitated (IP) with the α-SV and α-L antibodies or B) Incubated with glutathione-Sepharose beads (Beads) and the proteins analyzed by 7.5% SDS-PAGE. The positions of the L and GSTP-AO3 proteins are indicated.

protein was expressed (Fig. 11A, lane 3). When the L protein was expressed in the presence of GSTP-A03, the SV L protein still did not cobind with GSTP-A03 to the beads (Fig. 11B, lane 4) even though both proteins were expressed (Fig. 11A, lane 4). The other bands in Fig. 11A represent vaccinia proteins that were nonspecifically immunoprecipitated (lane 1). Thus the substituted domain did not function as a binding site.

The Chimera GSTP-A02 is Unable to Bind MV P

A second chimera was constructed that substituted the MV P/L binding domain from MV P into the SV L domain of SV P (Fig. 8). To determine whether or not this GSTP-A02 protein was capable of oligomerization, since in MV the binding sites for P and L overlap (Bankamp et al., submitted), the MV P protein was tested for its ability to bind to the chimera. No plasmids (-) or GSTP-A02 or MV P plasmids were transfected alone or together into VVT7 infected A549 cells. When GSTP-A02 was expressed alone, it bound to the glutathione-Sepharose beads (Fig. 12B, lane 2) as indicated. When the MV P protein was expressed alone, it did not bind to the beads (Fig. 12B, lane 3) even though the P protein was expressed (Fig. 12A, lane 3). The double asterisks mark the position of MV P which runs as a doublet. When the P protein was expressed in the presence of GSTP-A02, the MV P protein did not cobind with GSTP-A02 to the beads (Fig. 12B, lane 4) even though both proteins were expressed (Fig. 12A, lane 4)

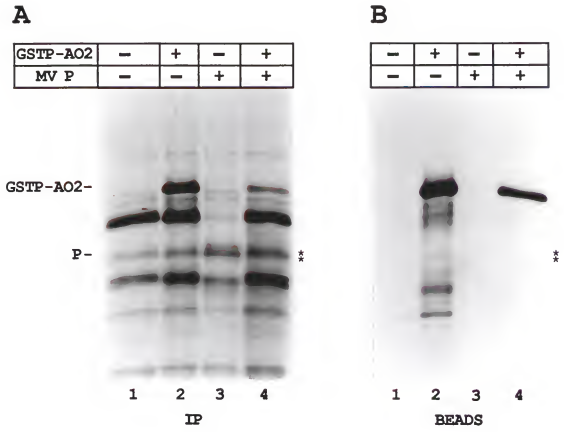


Fig. 12. Cobinding of the MV-P protein and the GSTP-AO2 fusion protein to glutathione-Sepharose beads. A549 cells infected with VVT7 were transfected with no plasmids (-), wt P plasmid (WT) or mutant P plasmids (1.7 µg) alone or together with GST-P plasmid (0.17 µg) as indicated. AT 5 h pt the cells were labeled for 5 h with Tran^{35}S-label and extracts prepared as described in Materials and Methods. Samples were A) Immunoprecipitated (IP) with the α-SV and α-L antibodies or B) Incubated with glutathione-Sepharose beads (Beads) and the proteins analyzed by 7.5% SDS-PAGE. The positions of the MV-P and GSTP-AO2 proteins are indicated.

suggesting no complex formed. The GSTP-AO2 protein, which retained the SV P oligomerization site aa 344-411, however, did retain the ability to bind SV P (Smallwood, unpublished data) suggesting that the protein is not misfolded. We did not perform similar experiments to test for binding of MV L to this chimera because the expression plasmids were not compatible.

Discussion

Our data provide evidence that the chimeric protein GSTP-AO3 and the GSTP-AO2 protein do not have the ability to bind the SV L or MV P proteins, respectively. There are minimally two possibilities for these negative results. First, while the identified binding domains are probably the primary sites for their specific protein interactions, these regions may also require additional regions outside of these domains in order to retain tight protein affinity. Second, it may be that by sequestering the chosen domains within these particular heterologous proteins, the domain is masked or is unable to retain a functional conformational state. In future experiments, it would seem beneficial to fuse the protein domain of interest at the end of a monomeric protein to allow for the best possible results as was previously shown for the MV P oligomerization site (Curran et al., 1995).

The L Binding Domain Charge-to-Alanine P mutants are Stably Expressed in Mammalian Cells

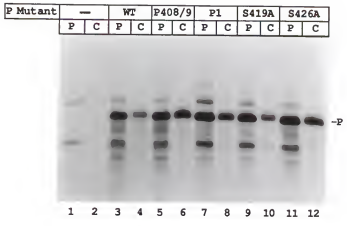
For the next aspect of P protein function, various charge-to-alanine P mutations, as outlined in Fig. 10, were constructed to test for the specific amino acids that are required in the L binding site of the Sendai P protein. As the first step we determined if the P mutants were stably expressed in cell culture. VVT7 infected A549 cells were transfected in duplicate with each of the wt or mutant P plasmids alone. At 5 h pt the cells were pulse labeled with duplicate dishes being chased overnight as described in Materials and Methods. The wt P protein was expressed in the pulse (Fig. 13A and B, lanes 3) and a significant fraction was stable to the chase (~30%) (Fig. 13A and B, lanes 4). Each of the P mutants was synthesized with a similar portion stable to the chase (Fig. 13A, lanes 5-12, and Fig. 13B, lanes 5-16). These data show that each of the P mutants is expressed so that functional studies can be performed.

Some of the Mutant P Proteins are Defective in *In Vitro* Transcription

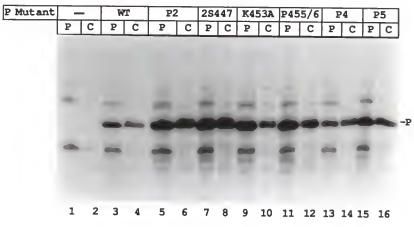
To establish the effects of such mutations within the polymerase complex, we first tested the mutants for their ability to synthesize viral RNA in an *in vitro* transcription assay. Thus, any defects noted may be ascribed only to the function of the polymerase and not to a possible encapsidation defect which would occur during replication.

Fig. 13. Pulse-chase analysis of the wt and charge-to-alanine mutant P proteins. A549 cells infected with VVT7 were transfected in duplicate with no plasmids (-), or the wt (WT) or mutant P plasmids (1.7 μg) as indicated at the top. At 5 h pt the cells were pulse labeled with Tran^{35}S-label and extracts prepared immediately (pulse, P) or at 16 h pt following a chase (C) as described in Materials and Methods. The viral proteins were immunoprecipitated with α-P peptide antibody and analyzed by 7.5% SDS-PAGE. The position of the P protein is indicated.

A


P Mutant
—
WT
P408/9
P1
S419A
S426A
P C P C P C P C P C P C
-P
1 2 3 4 5 6 7 8 9 10 11 12

B


P Mutant
—
WT
P2
2S447
K453A
P455/6
P4
P5
P C P C P C P C P C P C P C P C
-P
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

To test for the ability of the mutant P proteins to transcribe mRNA from a polymerase-free wt RNA-NP template, VVT7 infected A549 cells were cotransfected with wt or mutant P plasmids together with L plasmid at concentrations optimal for *in vitro* transcription (Horikami et al., 1992) and transcription was carried out as described in Materials and Methods. The wt P and L proteins were active in the synthesis of full length NP and P mRNA products (Fig. 14A, lanes 2 and 9 and Fig. 14B, lane 2), whereas no synthesis of mRNAs was detected in the absence of viral proteins (Fig. 14A, lanes 1 and 8 and Fig. 14B, lane 1). The P mutants S419A, S426A, and P455/6 all maintained significant transcriptional activity as compared to wild type, while mutants P1, P2, 2S447, K453A, and P5 showed a significant reduction in activity (Fig. 14A, lanes 4-7, 10 and 14B, lanes 3, 4, and 6, respectively). The mutant proteins P408/9 and P4 were most significantly impaired yielding very little transcription products (Fig. 14A, lane 3 and 14B, lane 6, respectively). In some experiments there are some small background products probably due to inefficient inactivation of cellular and vaccinia polymerase activity and/or inefficient nuclease treatment of the cell extracts prior to the start of transcription (Fig. 14A, lane 8 and Fig. 14B, lane 1). With the viral polymerase there are smaller products as well which may be due to either synthesis of the smaller M mRNA or, in some experiments, inefficient

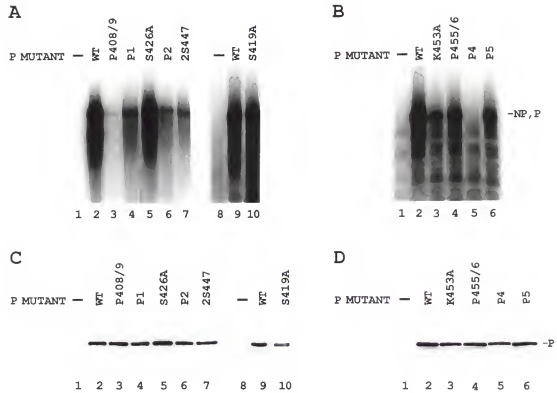


Fig. 14. *In vitro* transcription of the SV wt RNA-NP with the charge-to-alanine mutant P proteins. A and B) A549 cells were VVT7 infected and transfected with no plasmids (-) or cotransfected with wt or mutant P plasmids (1.5 µg) together with L plasmid (0.5 µg). Cytoplasmic cell extracts were prepared at 18 h pt and incubated with SV wt RNA-NP in the presence of [α-^{32}P]CTP. The transcripts were purified with the Quiagen RNeasy™ columns per manufacturer's protocol and analyzed on a 1.5% agarose-6 M urea gel. The relative positions of the NP and P transcripts are indicated. C and D) Immunoblot analysis using enhanced chemiluminescence on samples (10%) of the cytoplasmic extracts used for *in vitro* transcription with an α-P peptide antibody as described in Materials and Methods. The position of the P protein is indicated.

transcription by the viral polymerase causing premature termination of the transcripts. In addition, because the gel used to separate the mRNA species is not fully denaturing (6 M urea), smaller RNA species may retain secondary structure and thus may appear as extra products. A portion of the cell extracts (10%) used for *in vitro* transcription was used for immunoblot analysis as described in Materials and Methods. The wt and mutant P proteins were shown to be nearly equally expressed (Fig. 14C, lanes 2-10, and 14D, lanes 2-6). Figs. 14C and 14D correspond to Figs. 14A and 14B and show the wt and mutant proteins in similar lanes. Thus, mutagenesis of P amino acids throughout the L binding domain interestingly produced a spectrum of defective RNA synthesis phenotypes.

The Mutant P Proteins Assemble onto the Nucleocapsid Template

To ascertain whether the mutant P proteins, particularly those that were defective in some aspect of RNA synthesis, were capable of complex formation with L protein to form the viral polymerase, we utilized a nucleocapsid (NC) binding assay, which not only determined complex formation but simultaneously resolved whether these complexes could be assembled onto the NC template. It had been shown previously that the P-L polymerase binds through the P subunit (Horikami et al., 1995). VVT7 infected cells were transfected with no plasmids (-) or with wt or mutant P plasmids together with L plasmid. Cytoplasmic extracts were divided, and incubated in the presence or absence of NCs and pelleted through glycerol

as described in Materials and Methods. When the wt or mutant P proteins were coexpressed with L and pelleted in the absence of NC, no or only a faint amount of P or L was present in the pellet (Fig. 15A, lanes 3, 5, 7, 9, and 11; 15B, lanes 3, 5, and 7; and 15C, lanes 3, 5, 7, 9, and 11). In the presence of NC significantly more of both the P and L proteins were in the pellets for the wt and each mutant, and thus bound nucleocapsids (Fig. 15A, lanes 4, 6, 8, 10, and 12; 15B, lanes 4, 6 and 8; and 15C, lanes 4, 6, 8, 10, and 12). The other bands in some experiments are vaccinia proteins that nonspecifically pellet (Figs. 15B and C, lanes 2). These data suggest that all of these mutant P proteins are able to bind L to form a stable polymerase complex and to assemble this complex onto the nucleocapsid template in preparation for RNA synthesis.

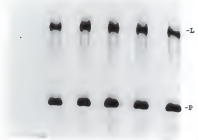
The Mutant P Proteins are Capable of Oligomerization

To ascertain whether or not the P mutants were capable of oligomerization, since this adjacent site was not mutated, radiolabeled wt or mutant P proteins were expressed in cells with a GST-P fusion protein, and the cobinding of P with GST-P to glutathione-Sepharose beads was used to measure P-P complex formation. Wt P protein expressed alone did not bind to the beads (Fig. 16A, B and C, lanes 2), however, when the P protein was expressed in the presence of GST-P, P cobound with GST-P to the beads showing complex formation (Fig. 16A, B, and C, lanes 4). Each of the mutants also cobound to

Fig. 15. Binding of the wt and charge-to-alanine mutant polymerase complexes to the wt nucleocapsid template. A549 cells infected with VVT7 were transfected with no plasmids (-) or cotransfected in duplicate with wt P (WT) or mutant P plasmids (5 μg) and wt L plasmid (5 μg) as indicated. Cells were radiolabeled for 16 h with Tran^{35}S-label, cytoplasmic cell extracts were prepared, and duplicate dishes pooled as described in Materials and Methods. The extract was divided, incubated in the absence or presence of polymerase-free wt RNA-NP (2.5 μg, NC), and pelleted through glycerol. The pellets were collected and analyzed by 7.5% SDS-PAGE. The positions of the P and L proteins are indicated.

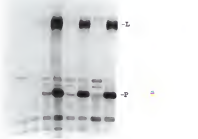
A

P Mutant	–	WT	P408/9	S419A	K453A	P455/6
L	–	+	+	+	+	+
NC	– +	– +	– +	– +	– +	– +



B

P Mutant	–	WT	P1	S426A
L	–	+	+	+
NC	– +	– +	– +	– +



C

P Mutant	–	WT	P2	S84A7	P4	P5
L	–	+	+	+	+	+
NC	– +	– +	– +	– +	– +	– +

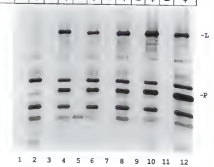
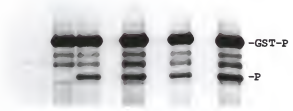


Fig. 16. Cobinding of the charge-to-alanine mutant P proteins and the GST-P fusion protein to glutathione-Sepharose beads. A549 cells infected with VVT7 were transfected with no plasmids (-), wt P plasmid (WT) or mutant P plasmids (1.7 μg) alone or together with GST-P plasmid (0.17 μg) as indicated. AT 5 h pt the cells were labeled for 16 h with Tran^{35}S-label and extracts prepared as described in Materials and Methods. Samples were incubated with glutathione-Sepharose beads (Beads) and the proteins analyzed by 7.5% SDS-PAGE. The positions of the P and GST-P proteins are indicated.

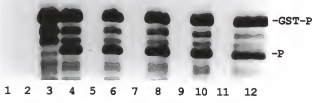
A

P Mutant	–	WT	–	WT	P408/9		P1		S426A	
GST-P	–	–	+	+	–	+	–	+	–	+



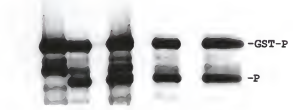
B

P Mutant	–	WT	–	WT	P2		S419A		K453A		P455/6	
GST-P	–	–	+	+	–	+	–	+	–	+	–	+



C

P Mutant	–	WT	–	WT	2S447		P4		P5	
GST-P	–	–	+	+	–	+	–	+	–	+

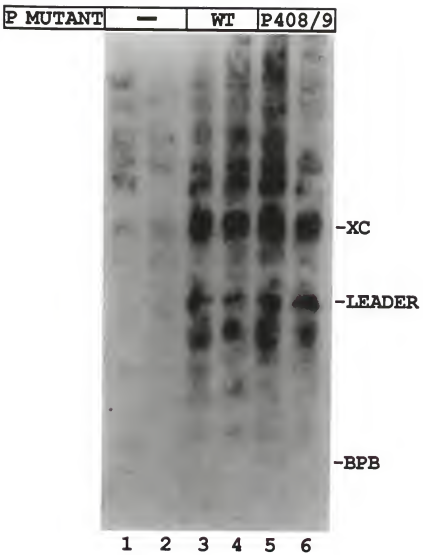


beads when expressed in the presence of the GST-P protein (Fig. 16A, lanes 6, 8, and 10; 16B, lanes 6, 8, 10, and 12; and 16C, lanes 6, 8, and 10), but not when expressed alone (Fig. 16A, lanes 5, 7, and 9; 16B, lanes 5, 7, 9, and 11; and 16C, lanes 5, 7, and 9). These data suggest that all the P mutants are capable of oligomerization. In summary, these protein interaction experiments show that the mutants are all able to form all of the known protein-protein interactions necessary for mRNA synthesis. The defect in some mutants, therefore, appears to be in the catalytic activity of the polymerase.

Mutant P408/9 is Able to Synthesize WT (+) Leader RNA

Since two of the P mutants, P408/9 and P4, displayed profound defects in mRNA synthesis yet maintained the wild type protein interactions, we wanted to test if the P408/9 protein could synthesize even the first transcription product, the 55 nt leader (*le+*) RNA from the SV wt RNA-NP template. VVT7-infected cells were cotransfected in duplicate with the wt or mutant P408/9 plasmid and L plasmid as for *in vitro* transcription except that the CTP concentration was increased to 1 mM in the absence of the radiolabeled nucleotide. The total RNA products were isolated and separated on an 8% polyacrylamide-8 M urea gel and analyzed by Northern blotting and hybridization to a uniformly labeled leader-NP riboprobe as described in Materials and Methods.

Fig. 17. Leader RNA synthesis with the mutant P408/9. VVT7-infected A549 cells were transfected in duplicate with no plasmids (-) or the wt L plasmid (0.5 µg) and either the wt P or the P408/9 plasmids (1.5 µg) as indicated. Cytoplasmic extracts were prepared as described for *in vitro* transcription in Materials and Methods and incubated with the wt SV RNA-NP. Total RNA products were separated on an 8% polyacrylamide-8 M urea gel, electroblotted onto Hybond N nitrocellulose, and detected with a ^{32}P-CTP uniformly labeled leader-NP riboprobe as described in Materials and Methods. The 55 nt leader RNA (*le+*) is indicated as well as the position of the xylene cyanol (XC) and bromophenol blue (BPB) dyes.


P MUTANT
—
WT
P408/9
-XC
-LEADER
-BPB
1 2 3 4 5 6

The wt polymerase synthesized *le+* RNA of 55 nt as well as longer products due to read through of the leader-NP gene boundary (Fig. 17, lanes 3 and 4) as reported earlier (Chandrika et al., 1995). Little *le+* RNA product is detected in the mock samples in which no viral proteins were expressed (Fig. 17, lanes 1 and 2). P408/9 synthesized the *le+* RNA at or near wild type levels (Fig. 17, lanes 5 and 6) indicating that the mutant polymerase is able to initiate RNA synthesis like the wt polymerase and which is, thus, not the defect in transcription. Preliminary evidence also indicates that P4 and K453A, which showed decreased synthesis in transcription, are able to synthesize *le+* as well (data not shown).

A Supplemental Role of the P Protein - Transcriptional Activity is Rescued by Wt P Protein for Some, but Not All of the P Mutants

Another possible role for P protein in RNA synthesis, aside from its activity within the polymerase complex and its necessity for encapsidation, has recently been described. Curran (1996) provides evidence that while part of the template associated P is bound as a P-L complex, the rest is bound to the template independently of L, and this latter form of P is also essential for mRNA synthesis. The exact mechanism of this supplemental function has yet to be delineated. To determine if the defects in activity could be due to an as yet undefined supplemental role of the P protein, rescue experiments were performed for several

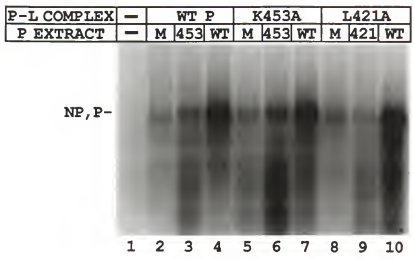
defective mutants.

First we tested K453A whose activity was decreased by 70% (Fig. 14B, lane 3). The wt or mutant polymerase complex was expressed at a ratio shown to be suboptimal (25%) for *in vitro* transcription (Fig. 18A, lane 2, 5 and 8). Cell extracts were prepared in 110 µl of SV RM salts and to 60% of these extracts was added either mock, wt P, or mutant P protein extracts (30%), where the P proteins were expressed alone as described in Materials and Methods. When wt P extract was added to wt P-L, transcriptional activity was rescued (Fig. 18A, lane 4). Rescue was not seen with the addition of a mock extract (Fig. 18A, lane 2), however, addition of the mutant K453A extract to wt P-L was able to partially rescue activity (~50%, Fig. 18A, lane 3). The mutant K453A, which is 30% as active as wild type (Fig. 14B, lane 3), could also be rescued to full activity by the addition of wt P extract (Fig. 18A, lane 7), but not by the addition of the mock extract (Fig. 18A, lane 5). The addition of the mutant K453A extract partially rescued the activity of the P mutant (~50%, Fig. 18, lanes 6). Immunoblot analysis showed that the P proteins were similarly expressed (Fig. 18B, lanes 3, 4, 6, and 7). The other mutant is described in Chapter 5.

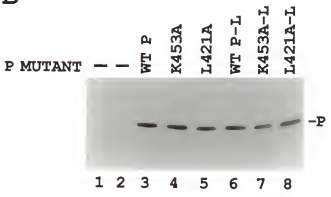
The two mutants, P408/9 and P4, which showed more severe defects in transcription, yielding little mRNA synthesis, were also tested for their ability to be rescued by the

Fig. 18. Rescue of *in vitro* transcription of the wt and the mutants K453A and L421A polymerases by the wt P protein. A) VVT7-infected cells were transfected with no plasmids or wt or mutant SV P plasmids alone (5 μg) and separate dishes cotransfected in duplicate with the same P plasmids and wt L plasmid at a 1:1 ratio (3 μg each). Cell extracts were prepared at 18 h pt and duplicate dishes pooled and 10% of each sample saved for immunoblot analysis (B). Cell extracts containing the wt or mutant viral polymerases were then divided into three 60 μl aliquots and to these were added 30 μl of a cell extract which contained either mock, wt P, or mutant P protein, where the P proteins were expressed alone. Cytoplasmic cell extracts were prepared at 18 h pt and incubated with SV wt RNA-NP in the presence of [α-^{32}P]CTP. The transcripts were purified with the Quiagen RNeasy™ columns per manufacturer's protocol and analyzed on a 1.5% agarose-6 M urea gel. The relative positions of the NP and P transcripts are indicated. B) Immunoblot analysis using enhanced chemiluminescence as described in Materials and Methods. The position of the P protein is indicated.

A


P-L COMPLEX
P EXTRACT
WT P
K453A
L421A
M
453
WT
421
NP,P-
1 2 3 4 5 6 7 8 9 10

B


P MUTANT
WT P
K453A
L421A
WT P-L
K453A-L
L421A-L
-P
1 2 3 4 5 6 7 8

addition of a wt P extract. However, rescue of these mutants to full activity by the addition of wt P extract did not occur (Figs. 19A and 20A, lanes 7), although wt P was active with the wt polymerase (Figs. 19A and 20A, lanes 4). Only a small amount of rescue could be seen with each of the mutants (~15-20%). Likewise, neither the mutant P408/9 nor P4 was successful in restoring full activity to the wt polymerase (Figs. 19A and 20A, lanes 3) or to each of the respective mutant polymerases (Figs. 19A and 20A, lanes 6). Interestingly, however, addition of either of the mutant P extracts to each polymerase gave a reproducibly slight decrease (~5%) in transcription when compared to the samples in which only mock extract was added. This may indicate that P408/9 and P4 contain dominant negative mutations. Immunoblot analysis showed that the proteins were similarly expressed (Figs. 19B and 20B, lanes 3-6).

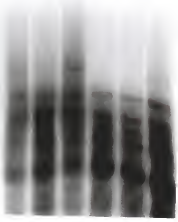
Sedimentation Analysis Provides Evidence for the Absence of Exchange between Free P and the P-L Complex

We wanted to test if rescue could occur because the free P exchanges with the P complexed with L. This was assayed by sedimentation analysis of ^{35}S-labeled P protein through 5-20% linear glycerol gradients either alone or after coexpression with L, or after incubation with unlabeled P-L extracts under transcription conditions. Gradient fractions (1 ml) were collected and 0.5 ml of each was immunoprecipitated with the α-P peptide antibody and

Fig. 19. Rescue of *in vitro* transcription of the wt and the P408/9 polymerases by the wt P protein. A) VVT7-infected cells were transfected with no plasmids (-) or wt or mutant SV P plasmids alone (5 μg) and separate dishes cotransfected in duplicate with the same P plasmids and wt L plasmid (3 μg each). Cell extracts were prepared at 18 h pt, duplicate dishes pooled and 10% of each sample saved for immunoblot analysis (B). Cell extracts containing the wt or mutant viral polymerases were then divided into three 60 μl aliquots and to these were added 30 μl of a cell extract which contained either mock, wt P, or mutant P protein, where the P proteins were expressed alone. Cytoplasmic cell extracts were prepared at 18 h pt and incubated with SV wt RNA-NP in the presence of [α-^{32}P]CTP. The transcripts were purified with the Quiagen RNeasy™ columns per manufacturer's protocol and analyzed on a 1.5% agarose-6 M urea gel. The relative positions of the NP and P transcripts are indicated. B) Immunoblot analysis using enhanced chemiluminescence as described in Materials and Methods. The position of the P protein is indicated.

A

P-L COMPLEX	—	WT P			P408/9		
P EXTRACT	—	M	408	WT	M	408	WT



B

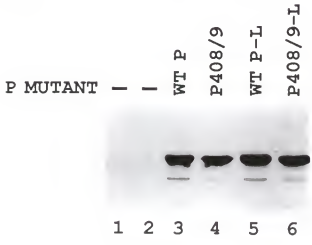


Fig. 20. Rescue of *in vitro* transcription of the wt and the P4 polymerases by the wt P protein. A) VVT7-infected cells were transfected with no plasmids (-) or wt or mutant SV P plasmids alone (5 µg) and separate dishes cotransfected in duplicate with the same P plasmids and wt L plasmid (3 µg each). Cell extracts were prepared at 18 h pt and duplicate dishes pooled and 10% of each sample saved for immunoblot analysis (B). Cell extracts containing the wt or mutant viral polymerases were then divided into three 60 µl aliquots and to these were added 30 µl of a cell extract which contained either mock, wt P, or mutant P protein, where the P proteins were expressed alone. Cytoplasmic cell extracts were prepared at 18 h pt and incubated with SV wt RNA-NP in the presence of [α-^{32}P]CTP. The transcripts were purified with the Quiagen RNeasy™ columns per manufacturer's protocol and analyzed on a 1.5% agarose-6 M urea gel. The relative positions of the NP and P transcripts are indicated. B) Immunoblot analysis using enhanced chemiluminescence as described in Materials and Methods. The position of the P protein is indicated.

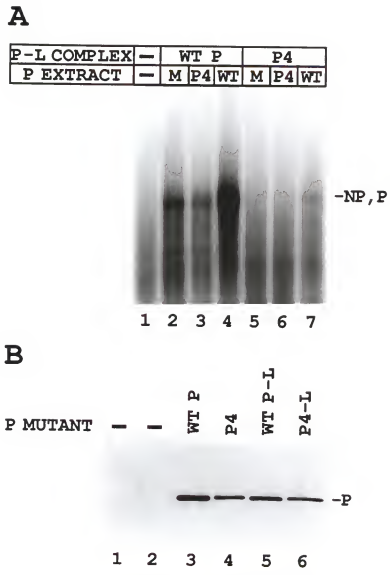
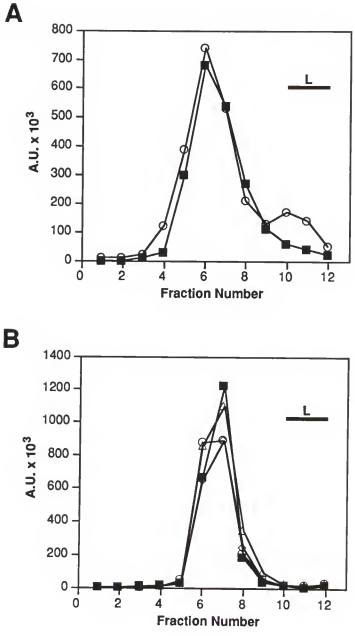

A
P-L COMPLEX — WT P P4
P EXTRACT — M P4 WT M P4 WT
-NP,P
1 2 3 4 5 6 7
B
P MUTANT — — WT P P4 WT P-L P4-L
-P
1 2 3 4 5 6

Fig. 21. Analysis of exchange between the wt or mutant P protein and the wt polymerase complex. A) VVT7-infected A549 cells were transfected with the wt P plasmid (5 μg) alone (■) or together with the L plasmid (○), and labeled with Tran^{35}S-label from 5-16 h pt. Cytoplasmic extracts were fractioned on a glycerol gradient, the proteins immunoprecipitated with the α-SV and α-L antibodies, analyzed by 7.5% SDS-PAGE, quantitated on the phosphoimager, and plotted in arbitrary units as described in Materials and Methods. The position of sedimentation of the L protein is indicated by the bar. B) The amount of label in P protein in each fraction of gels Fig. 22A-D was quantitated on the phosphoimager and plotted in arbitrary units. The P proteins are indicated as follows: wt P alone (■) and wt P (○), P408/9 (Δ), and L421A (◇) after incubation with the wt polymerase complex. The position of sedimentation of the L protein is indicated by the bar.


A
800
700
600
500
400
300
200
100
0
A.U. x 10³
L
0
2
4
6
8
10
12
Fraction Number
B
1400
1200
1000
800
600
400
200
0
A.U. x 10³
L
0
2
4
6
8
10
12
Fraction Number

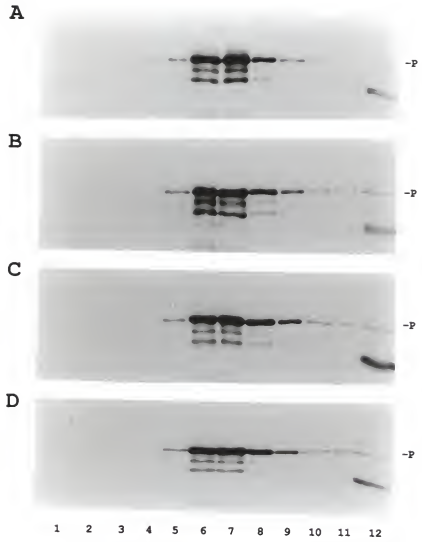
fractioned by SDS-PAGE. Each fraction was quantitated on the phosphorimager as described in Materials and Methods and plotted as shown in Figs. 21A and B. As the control for complex formation, P protein expressed alone sedimented presumably as a trimer (Curran et al., 1996) primarily in fractions 5-8, however, when the P protein was expressed with L a significant amount of P now cosedimented with L in fractions 10-12 (gels not shown, Fig. 21A).

When wt P and P408/9 were sedimented after incubation with the unlabeled P-L polymerase complex, the peak levels of P were only seen in fractions 6-8 (Figs. 22B-D) identical to the sedimentation profile of P alone (Figs. 22 and Fig. 21B). Thus, no shift was detected into fractions 10-12 which would indicate exchange and complex formation with L (Fig. 21B). These data suggest that exchange between a free pool of P protein and that associated with the L protein does not occur during rescue.

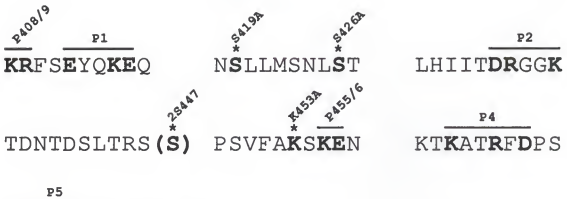
Summary

In conclusion, in an effort to identify the amino acids within the P protein important for complex formation with L protein and for function, 10 charged-to-alanine mutants were constructed in the region spanning the L binding site. We first assessed the ability of the each of the mutants to transcribe viral mRNA and found a spectrum of wt and defective phenotypes as summarized in Fig. 23B. To achieve full transcriptional activity, the P protein must complex

Fig. 22. Sedimentation analysis of wt P and the mutants P408/9 and L421A after incubation with the wt polymerase complex. VVT7-infected cells were transfected with the plasmid (5 μg): wt P (A and B); P408/9 (C); or L421A (D) and labeled with Tran^{35}S-label from 5-16 h pt as described in Materials and Methods. Cytoplasmic extracts were prepared. B-D) Prior to sedimentation samples B-D were incubated with an equal volume of an unlabeled wt polymerase complex cell extract for 1 h under *in vitro* transcription conditions as described in Materials and Methods. The samples were separated on a 5-20% (vol/vol) glycerol gradient and 1 ml fractions collected from the top. One half of the sample was immunoprecipitated with the α-P peptide antibody and analyzed by 7.5% SDS-PAGE. Sedimentation was from left to right. The position of the P protein is indicated.


A
B
C
D
-P
-P
-P
-P
1 2 3 4 5 6 7 8 9 10 11 12

A



B

P PROTEIN	*IN VITRO* TRANSCRIPTION	RESCUE
WT P	++++	++++
P408/9	+	++
P1	++	ND
S419A	++++	ND
S426A	++++	ND
P2	++	ND
2S447	++	ND
K453A	++	++++
P455/6	+++	ND
P4	+	+
P5	++	ND

Fig. 23. A summary of the *in vitro* transcription and rescue data of the mutant P proteins. A) A schematic of the P protein and the aa sequences from 408 to 479 of the charge-to-alanine P mutants. Mutant P proteins are highlighted by bold lettering. Single changes are indicated by an asterisk while grouped changes are indicated by an overline. B) *In vitro* transcription and rescue with wt P of the mutant P proteins from Figs. 14, 18, 19, and 20. The plus signs refer to the amount of transcription in multiple experiments (3-6) as compared to wt P, where ++++ refers to >85%; +++, 50-85%; ++, 15-50%; and +, <15%.

with the L protein to form the active RNA-dependent RNA polymerase, assemble this complex onto the template and then synthesize viral mRNA utilizing the proper intergenic transcriptional signals. To determine if the defective phenotypes were a consequence of a defect in the protein-protein interactions, a NC binding assay and P oligomerization assay were employed. All of the mutants retained the wild type protein-protein interactions necessary for biological activity.

These data also provide evidence for the first time that P may have a functional role in RNA synthesis. To determine if the defects in transcription were due to an abrogation of polymerase function or possibly to a defect in a supplemental role of the P protein, rescue experiments were performed. The data showed that rescue could be accomplished for mutants K453A, but not for P408/9 or P4 (Figs. 18-20A and 23). These results indicate that mutants which are rescued may be defective in a supplemental role of P, while the mutants which are not rescued may represent more defects in catalytic activity. These data also strongly indicate that the phenomenon of rescue does not occur by an exchange process between free P and that which is associated with the polymerase (Figs. 21 and 22). An in depth discussion of these data is presented in Chapter 6.

CHAPTER 5
IDENTIFICATION OF HYDROPHOBIC AMINO ACIDS
IMPORTANT FOR L BINDING

Introduction

To ascertain specific amino acids in the P protein important for binding L, we initially changed charged residues to alanine within the L binding domain (aa 412-479) to determine if complex formation was electrostatic in nature. We presented evidence that all of the charge-to-alanine mutants maintained their ability to complex with L protein, even though some showed a loss of function (Chapter 4). To further investigate the P-L interaction with respect to the specific amino acids involved within the P protein, efforts were directed next at determining if instead the interaction was due primarily to a hydrophobic domain. There are four classical energies involved in protein-protein interactions as well as protein folding and stability. These are electrostatic forces, hydrophobic forces, hydrogen bonding, and Van der Waals forces. Hydrogen bonds are often established between the peptide backbones of interacting proteins as well as between the various amino acids residues, and thus, these interactions occur in a broad and frequent manner and would be difficult to characterize. Van der Waals forces are the attractive forces which exist between

molecules in very close proximity and while, they are known to play a significant role overall in protein-protein interactions, due to their small nature, they would be difficult to measure in an individual manner. Subsequently, our choice to analyze the hydrophobic residues in the L binding domain is the next logical step in determining the nature of the P-L interaction. In addition, through mutation of hydrophobic amino acids, Myers et al. (1997) identified specific residues within the central conserved region (CCR) of the Sendai virus NP protein which were important for either NP-NP or NP-RNA interactions as well as for genome replication.

To determine the effect of hydrophobic forces in P-L complex formation, we selected a hydrophobic-to-alanine mutagenesis approach. As for the charge-to-alanine mutations, we chose this method because of the likelihood of producing stable mutants capable of exhibiting altered phenotypes. We targeted 5 single aa of the P protein within the proximal portion of the L binding domain from aa 408 to 445 for mutagenesis as shown in Table 3 of Materials and Methods. Only the proximal portion was chosen because published evidence showed that L binding occurs from aa 412-445 (Curran et al., 1994). These amino acids were chosen due to their hydrophobic nature as well as their conservation among the parainfluenza- and morbilliviruses (Fig. 24A). We will present evidence that all of these mutants maintain

their ability to complex with L protein, but two proved defective in RNA synthesis.

Results

The Hydrophobic-to-Alanine P Mutants are Stably Expressed in Mammalian Cells

Five mutations as indicated in Fig. 24A were constructed to test for functional amino acids in the L binding site of the Sendai P protein. As the first step we determined if the P mutants were stably expressed in cell culture. VVT7 infected A549 cells were transfected in duplicate with each of the wt or mutant P plasmids alone. At 5 h pt the cells were pulse labeled with duplicate dishes being chased overnight as described in Materials and Methods. The wt P protein was expressed in the pulse (Figs. 24B and C, lanes 3) and a significant fraction was stable to the chase (~35%) (Figs. 24B and C, lanes 4). Each of the P mutants was synthesized with a similar portion stable to the chase (Fig. 24B, lanes 5-10, and Fig. 24C, lanes 5-8). These data show that each of the P mutants is expressed so that functional studies can be performed.

Two of the Mutant P Proteins are Defective in *In Vitro* Transcription

To establish the effect of each mutation within the polymerase complex, we first tested the mutants for their ability to synthesize viral RNA in an *in vitro* transcription assay. Thus, any defects noted may be ascribed only to the

Fig. 24. Sequence comparison of the parainfluenza- and morbillivirus P proteins and pulse-chase analysis of the wt and hydrophobic-to-alanine mutant P proteins. A) Sequence comparison of conserved hydrophobic residues between the parainfluenza- and morbillivirus P proteins from amino acids (aa) 408 to 447. The most highly conserved aa are highlighted by bold lettering and by an asterisk placed above the column. B and C) A549 cells infected with VVT7 were transfected in duplicate with no plasmids (-), or the wt (WT) or mutant P plasmids (1.7 μg) as indicated at the top. At 5 h pt the cells were pulse labeled with Tran^{35}S-label and extracts prepared immediately (pulse, P) or at 16 h pt following a chase (C) as described in Materials and Methods. The viral proteins were immunoprecipitated with α-P peptide antibody and analyzed by 7.5% SDS-PAGE. The position of the P protein is indicated.

A

```
          AA408                                          AA447
SV-P      KRFSEYQK EQNSLLMSNL STLHITDRG GKTDNTDSLTRS
hPIV3-P   RRLIENQR EQLSLITSLI SNLKIMTERG GKKDQNESNERV
bPIV3-P   RRLIENQR EQLSLITSLI SNLKIMTERG GKKDQPENSGRT
MV-P      ........ ...STLEGHL SSIMIAIPGL GKDPNDPTADVE
RV-P      ........ ...STIEGHL SSFMIAIPGF GKDPNDPTADVD
CDV-P     ........ ...STIEGHL SSIMIAIPGF GKDTGDPTANVD
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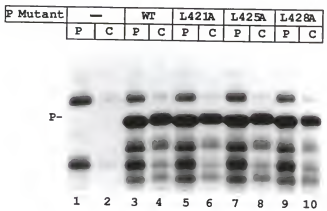
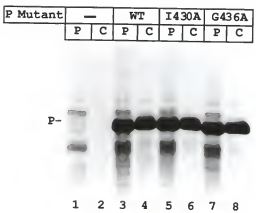
B



C



function of the polymerase and not to a possible error in encapsidation occurring during replication. To test for the ability of the mutant P proteins to transcribe mRNA from a polymerase-free wt RNA-NP template, VVT7 infected A549 cells were cotransfected with wt or mutant P plasmids together with L plasmid at concentrations optimal for *in vitro* transcription (Horikami et al., 1992) and transcription was carried out as described in Materials and Methods. The wt P and L proteins were active in the synthesis of full length NP and P mRNA products (Figs. 25A and B, lanes 2), whereas no synthesis of the full length products was detected in the absence of viral proteins (Figs. 25A and B, lanes 1). The P mutants L428A, I430A, and G436A all synthesized full length NP and P mRNAs at or near wild type levels, while mutants L421A and L425A showed a significant reduction (~70%) in activity (Fig. 25A, lanes 3-5, and 24B, lanes 3 and 4). A portion of the cell extracts was used for immunoblot analysis as described in Materials and Methods and the wt and mutant P proteins were shown to be equally expressed (Fig. 25C, lanes 2-5, and 25D, lanes 2-4). Figs. 25C and 25D correspond to Figs. 25A and 25B, respectively and show the wt and mutant proteins in similar lanes. Thus, mutagenesis of hydrophobic P amino acids within the L binding domain produced only two mutants which were significantly reduced in activity.

The Mutant P Proteins Assemble onto the Nucleocapsid Template

To ascertain whether the mutant P proteins, particularly

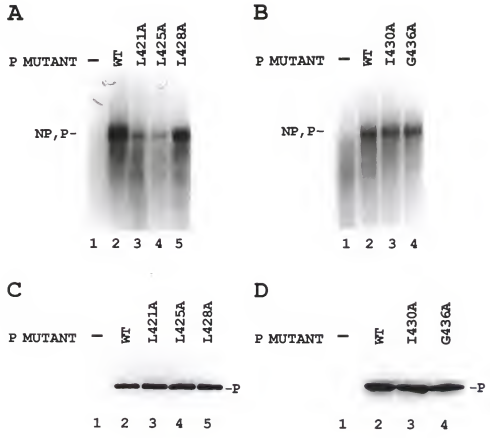


Fig. 25. *In vitro* transcription of the SV wt RNA-NP with the hydrophobic-to-alanine mutant P proteins. A and B) A549 cells were VVT7 infected and transfected with no plasmids (-) or cotransfected with wt or mutant P plasmids (1.5 μg) together with L plasmid (0.5 μg). Cytoplasmic cell extracts were prepared at 18 h pt and incubated with SV wt RNA-NP in the presence of [α-^{32}P]CTP. The transcripts were purified with the Quiagen RNeasy™ columns per manufacturer's protocol and analyzed on a 1.5% agarose-6 M urea gel. The relative positions of the NP and P transcripts are indicated. C and D) Immunoblot analysis using enhanced chemiluminescence on samples (10%) of the cytoplasmic extracts used for *in vitro* transcription with an α-P peptide antibody as described in Materials and Methods. The position of the P protein is indicated.

the two that were defective in RNA synthesis, were capable of complex formation with L protein to form the viral polymerase, we utilized a nucleocapsid (NC) binding assay, which not only determined complex formation but simultaneously resolved whether these complexes could be assembled onto the NC template. VVT7 infected cells were transfected with no plasmids (-) or with wt or mutant P plasmids together with L plasmid. Cytoplasmic extracts were divided, and incubated in the presence or absence of NCs and pelleted through glycerol as described in Materials and Methods. When the mutant P proteins were coexpressed with L and pelleted in the absence of NC, no or only a small amount of P or L was present in the pellet (Fig. 26, lanes 4, 6, 8, 10, and 12). It was previously shown that the wt P and L proteins also do not pellet in the absence of NC (Fig. 5B, lane 3 and Figs. 15A-C, lanes 3). In the presence of NC significantly more of both the P and L protein were in the pellets for the wt and each mutant, and thus bound nucleocapsids (Fig. 26, lanes 3, 5, 7, 9, 11, and 13). These data suggest that all of these mutant P proteins are able to form a stable polymerase complex and to assemble this complex onto the nucleocapsid template in preparation for RNA synthesis.

The Mutant P Proteins are Capable of Oligomerization

To ascertain whether or not the P mutants were capable of oligomerization, radiolabeled wt or mutant P proteins were

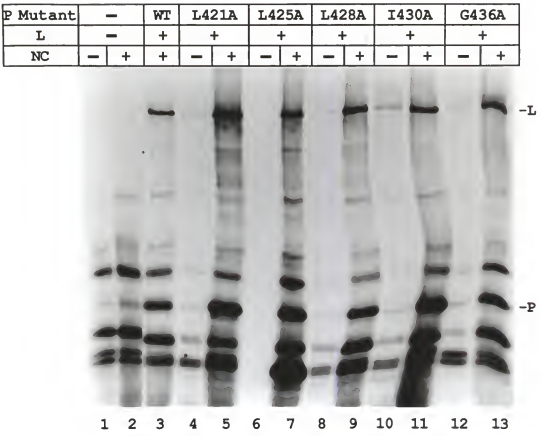


Fig. 26. Binding of the wt and hydrophobic-to-alanine mutant polymerase complexes to the wt nucleocapsid template. A549 cells infected with VVT7 were transfected with no plasmids (–) or cotransfected in duplicate with wt P (WT) or mutant P plasmids (5 μg) and wt L plasmid (5 μg) as indicated. Cells were radiolabeled for 16 h with Tran^{35}S-label, cytoplasmic cell extracts were prepared, and duplicate dishes pooled as described in Materials and Methods. The extract was divided, incubated in the absence or presence of polymerase-free wt RNA-NP (2.5 μg, NC), and pelleted through glycerol. The pellets were collected and analyzed by 7.5% SDS-PAGE. The positions of the P and L proteins are indicated.

expressed in cells with a GST-P fusion protein, and the cobinding of P with GST-P to glutathione-Sepharose beads was used as a measure of P-P complex formation. Wt P protein was expressed alone (data not shown) but did not bind to the beads (Fig. 27, lane 2), however, when the P protein was expressed in the presence of GST-P, P cobound with GST-P to the beads showing complex formation (Fig. 27, lane 4). Each of the mutants also cobound to beads when expressed in the presence of the GST-P protein (Fig. 27, lanes 6, 8, 10, 12, and 14), but not when expressed alone (Fig. 16A, lanes 5, 7, 9, 11, and 13). These data suggest that all the P mutants are capable of oligomerization. These proteins are thus able to form all of the known protein-protein interactions necessary for mRNA synthesis, even though the biological data suggest that two of these residues are important for catalytic activity.

Transcriptional Activity is Rescued by Wt P Protein for P Mutant L421A

To determine if the defects in activity could be due to an as yet undefined supplemental role of the P protein as described in Chapter 4, a rescue experiment was performed on L421A. The wt or mutant polymerase complex was expressed at levels shown to be suboptimal (25-30%) for *in vitro* transcription (Chapter 4, Fig. 18, lanes 2 and 8). Cell extracts were prepared in 110 µl of SV RM salts and to 60% of these extracts was added either mock, wt P, or mutant P

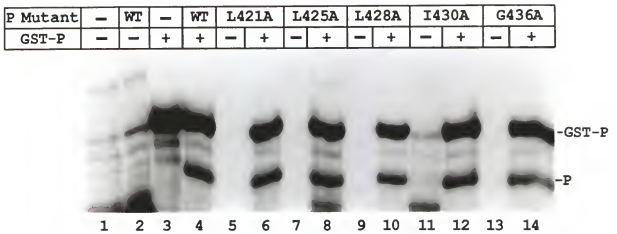


Fig. 27. Cobinding of the hydrophobic-to-alanine mutant P proteins and the GST-P fusion protein to glutathione-Sepharose beads. A549 cells infected with VVT7 were transfected with no plasmids (-), wt P plasmid (WT) or mutant P plasmids (1.7 μg) alone or together with GST-P plasmid (0.17 μg) as indicated. At 5 h pt the cells were labeled for 16 h with Tran^{35}S-label and extracts prepared as described in Materials and Methods. Samples were incubated with glutathione-Sepharose beads (Beads) and the proteins analyzed by 7.5% SDS-PAGE. The positions of the P and GST-P proteins are indicated.

protein extracts (30%), where the P proteins were expressed alone as described in Materials and Methods. When wt P extract was added to wt P-L, transcriptional activity was rescued to 100% of wt activity (Fig. 18, lane 4). Addition of the mutant L421A extract to wt P-L was able to partially rescue activity (from 30% to ~50%, data not shown). L421A, which was 30% as active as wild type, could also be rescued to full activity by the addition of wt P extract (Fig. 18, lane 10), but not by the addition of mock extract (Fig. 18, lane 8), although addition of the mutant extract partially rescued the activity (from 25% to ~50%, Fig. 18, lane 9). Immunoblot analysis showed that the proteins were all expressed at similar levels (Fig. 18B).

Summary

In an effort to identify the amino acids within the P protein important for complex formation with L protein and for function, 5 hydrophobic-to-alanine mutants were constructed in the proximal region of the L binding site. We first assessed the ability of the each of the mutants to transcribe viral mRNA and found two, L421A and L425A, to be significantly reduced in transcription as summarized in Fig. 28B. Interestingly, the completely conserved aa I430A and G436A were not required for activity. To achieve full transcriptional activity, the P protein must complex with the L protein to form the active RNA-dependent RNA polymerase, assemble this complex onto the template and then synthesize

A

```
          AA408                                          AA447
                                 *   *   * *     *
   SV-P   KRFSEYQK EQNSLLMSNL STLHIITDRG GKTDNTDSLTRS
hPIV3-P   RRLIENQR EQLSLITSLI SNLKIMTERG GKKDQNESNERV
bPIV3-P   RRLIENQK EQLSLITSLI SNLKIMTERG GKKDQPENSGRT
   MV-P   ........ ...STLEGHL SSIMIAIPGL GKDPNDPTADVE
   RV-P   ........ ...STIEGHL SSFMIAIPGF GKDPNDPTADVD
  CDV-P   ........ ...STIEGHL SSIMIAIPGF GKDTGDPTANVD
```

B

P PROTEIN	*IN VITRO* TRANSCRIPTION	RESCUE
WT P	++++	++++
L421A	++	++++
L425A	++	**ND**
L428A	++++	**ND**
I430A	++++	**ND**
G436A	++++	**ND**

Fig. 28. A summary of the *in vitro* transcription and rescue data for the hydrophobic-to-alanine mutants. A) Sequence comparison of conserved hydrophobic residues between the parainfluenza- and morbillivirus P proteins from amino acids (aa) 408 to 447. The most highly conserved aa are highlighted by bold lettering and by an asterisk placed above the column. B) *In vitro* transcription and rescue with wt P of the mutant P proteins from Figs. 18 and 25. The plus signs refer to the amount of transcription in multiple experiments (3-5) as compared to wt P, where ++++ refers to >85%; +++, 50-85%; ++, 15-50%; and +, <15%.

viral mRNA utilizing the proper intergenic transcriptional signals. To determine if the defective phenotypes were a consequence of a defect in the protein interactions, a NC binding assay and P oligomerization assay were employed. All of the mutants retained the protein-protein interactions necessary for biological activity. These data also provide evidence that the mutant P protein L412A may be rescued to wild type activity by wt P protein. Thus, the defect in transcription of this mutant may possibly be due to a defect in an undefined supplemental role of the P protein. For a discussion of the complete sets of data, see Chapter 6.

CHAPTER 6
DISCUSSION

The RNA-dependent RNA polymerases of the negative-strand RNA viruses are unique in that they recognize the viral genome only in the encapsidated form and must interact with this template in an intricate manner to control gene expression and viral replication. Central to the understanding of how RNA synthesis occurs on these unusual templates lies the multifunctional P protein. P must interact with the L protein to provide the viral polymerase, with the nucleocapsid template to bind the polymerase, with the NP protein to provide a substrate for genome encapsidation, and with itself. Extensive deletional analyses has provided much of the current data on the P protein, and although it has been instrumental in defining regions of the protein involved in its different protein-protein interactions as well as promoting a clearer understanding of its role in nascent chain assembly, the role of the P protein within the polymerase has remained elusive.

The P protein has been shown to be required for binding of the P-L complex to NCs (Horikami and Moyer, 1995) and to promote the stability of the L protein perhaps by helping it to fold properly. In the absence of P, the L protein half

life is 1.2 h, whereas in the P-L complex it is 15 h (Horikami et al., 1997). Deletion analysis of the Sendai virus P protein has identified the region from aa 412-479 (Smallwood et al., 1994) or aa 412-445 (Curran et al., 1994) as containing the L binding site. This domain resides discreetly between the NC binding (aa 345-411 and 479-568) and the P oligomerization (aa 345-411) domains (Ryan et al., 1988, 1990; Curran et al., 1995b). In an effort to identify the amino acids within the P protein important for complex formation with L protein and for function, 10 charged-to-alanine and 5 hydrophobic-to-alanine mutants were constructed in the region spanning aa 408-479.

We first assessed the ability of the each of the mutants to transcribe viral mRNA. To achieve full transcriptional activity, the P protein must complex with the L protein to form the active RNA-dependent RNA polymerase, assemble this complex onto the template and then synthesize viral mRNA utilizing the proper intergenic transcriptional signals. Of all the mutants tested, the mutant P proteins S419A, S426A, P455/6, L428A, I430A, and G436A were able to synthesize full length viral mRNAs at or near wild type levels, while the mutants P1, P2, 2S447, K453A, P5, L421A and L425A were significantly reduced (<50%) in transcription (Figs. 23 and 28). The mutant proteins P408/9 and P4 were the most impaired yielding little or no transcription products. Thus sites essential for activity are interspersed thoughout the

region. This was unexpected since Curran et al. (1994) proposed that only the residues from 412-445 were necessary for binding and P function. Clearly these data show that the distal portion of this domain, which lies outside Curran's L binding site, is critical for polymerase function in mRNA synthesis.

To determine if the defective phenotypes were a consequence of a defect in the protein interactions, a NC binding assay and P oligomerization assay were employed. All of the mutants retained the wildtype protein-protein interactions necessary for biological activity, yet some had lost transcriptional activity. Altogether the mutant P proteins which were significantly reduced in transcription were composed of 16 charged amino acids, but only 2 hydrophobic amino acids. Mutants P408/9 and P4, which were severely defective, being less than 15% as active as wild type, were composed of two (lysine and arginine) and three charged residues (lysine, arginine, and aspartate), respectively.

Thus, we propose that the L binding domain may be folded in such a way that the residues important for complex formation are spaced throughout a more complex tertiary structure which represent a surface involved in electrostatic interactions. For example, since P408/9 and P4 are located at each end of the proposed binding domain, it may be hypothesized that this region folds or loops around in such a

manner that the amino acids of these mutants are in close proximity and provide a surface for interaction. Thus, we would predict that mutation of both groups together may yield a protein incapable of binding L. It has been shown, however, that deletion of aa 446-479, which contains P4, still provides enough surface for L binding to occur (Curran et al., 1994). Several models are possible for why individual or small groups of changes and even a larger deletion do not alter binding. First each individual mutation may have a reduced affinity for L which is undetectable in our assays, but which leads to the transcriptional defects. Second binding may occur incorrectly so that the L protein may not fold properly and thus, be inactive.

The mutational studies of the hydrophobic residues were not as extensive as those for the charged amino acids, and it is possible that if more residues were mutated and in groups instead of individually, there would have been an increase in the percentage of defective phenotypes providing results similar to that for the charged mutants. However, upon close inspection of the binding domain from aa 408 to 479, it is clear that there is a higher percentage of charged as compared to hydrophobic residues. The literature states that there are 1.6 times the number of hydrophobic to charged residues available for incorporation into protein, but for P there are only 0.83 as many hydrophobic to charged amino

acids within the domain studied. Thus, the charged residues are probably more important. The decrease in activity of L421A and L425A may be due to a defect in protein folding and/or stability in this region. Such a defect may not be large enough to lead to a substantial decrease in either protein production, and thus would appear wild type in pulse-chase analyses as did these mutants (Fig. 24B), or in protein-protein interactions. However, it is clear that hydrophobic interactions play an important role in the protein conformation.

Hydrophobic interactions are attractions, frequently by van der Waals forces, in aqueous medium between apolar and amphipathic molecules. Among others, they have been recognized as important for the stabilization of folded ribonuclease T1 and α-helices in peptides. Alternatively, an increase in hydrophobic character in some peptides, including hemoglobin, has been observed to promote instability (for a review, see Tsai et al., 1996; Cserhati and Szogyi, 1995). This latter example may explain some of the defective phenotypes of the charge-to-alanine mutants. Overall, the relative importance of any residue for the strength of a protein-protein interaction depends ultimately on many factors, including steric correspondence, conformational change, folding state of the interacting molecular species, environmental pH, and salt concentrations. Although for reasons stated previously we do believe the P-L interaction

to be primarily electrostatic, the character of all the forces involved cannot unequivocably be determined at this time. To further investigate this problem, P mutants could be constructed which combine several of the above defective mutants and tested for P-L interactions. Also studies could be performed in which the pH and salt concentrations were altered, as well as determining the effect of adding detergents to the protein complex. Ultimately these studies may provide a better understanding of the required interactions.

Although we did not define single amino acids important for binding, our analyses have led to an unexpected and interesting observation. As mentioned previous to these studies, the P protein was hypothesized to have many important roles such as for the proper conformational folding in the stable expression of L and for binding of the P-L complex to the NC. However, it has not been shown to contain any catalytic function. It is believed that the L protein contains most of the activities needed for RNA synthesis and processing, although there is little direct experimental evidence on this point from studies of the paramyxoviruses. Due to its high degree of phosphorylation, it has been suggested that P may act as a regulatory factor (Kolakofsky et al., 1991) or possibly to function as a regulatory factor for RNA elongation, as was postulated for VSV (De and Banerjee, 1985). However, phosphorylation studies have been

frequently contradictory in nature in VSV. Phosphorylation is important for the function some P proteins, however, such as for VSV P oligomerization (Gao and Lenard, 1995b). Mutation of the major phosphorylation site, S249, as well as mutations of the putative minor sites within the L binding domain, S419 and S426, proved unessential for Sendai P function (Chapters 3 and 4) and thus, it remains unclear whether phosphorylation is relevant in this virus.

The only other studies strictly involving function of P within the P-L complex has been the analysis of the N-terminus. The hypervariable, acidic N-terminus of SV P has been the center of much study due to it similarities to the acidic activation domains of cellular transcription factors (Curran et al., 1994). In fact, the required N-terminus of VSV P could be functionally substituted by tubulin (Chattopadhey and Banerjee, 1988) and purified tubulin stimulated SV and MV mRNA synthesis (Moyer et al., 1986, 1990). Thus functional studies have been sparse. For the first time, we have provided evidence through site-directed mutations that the paramyxovirus P proteins may have a catalytic role important for polymerase function.

We have constructed many P mutants which retain the wild type protein interactions, yet are defective to varying degrees in biological activities. It may be that the mutations disrupted the proper conformation of the polymerase, as discussed, therefore, interrupting function.

More interestingly, these phenotypes could be explained in an alternate manner. Each of these mutations were constructed within the proposed L binding domain. It is possible to imagine that the P and L proteins interact to form the catalytic domain necessary for polymerization of RNA. Indeed, the P protein is known to bind the complex to the viral template. One may visualize that P is located between the template and L protein and thus contributes to an area of catalytic activity. Indeed Ryan and coworkers (1990, 1991) speculated that the L binding domain from aa 412-478 could loop out from between the two NC binding domains allowing such an interaction to take place. However, it would be difficult to prove this hypothesis since the P and L proteins cannot be dissected separately and must be coexpressed to form an active polymerase.

These data did present another exciting avenue of exploration. While not defining a direct catalytic role for the P protein within the polymerase, they provide evidence for the first time that P may have a functional role in RNA synthesis. We wanted to determine if the defects in transcription were due to an abrogation of polymerase function or possibly to a defect in a supplemental role of the P protein as Curran (1996) suggested. This supplemental role was observed to involve, in part, the binding of additional copies of P independent of L to the NC template. Cell extracts optimized for mRNA synthesis contain a large

molar excess of P, up to 70-fold (De and Banerjee, 1985) and immunoelectron microscopy of intracellular SV holo-nucleocapsids showed that P is found bound to the template both independent of and together with L, whereas L is only found associated with P (Portner et al., 1988). In VSV studies it has been proposed that only one P trimer appears to be bound per L (Gao and Lenard, 1995a, b). While recent evidence suggests that SV P can also trimerize (Curran et al., 1995), it remains unclear what form it takes within the polymerase complex or when bound to the template independent of L. Thus there is clearly more P than is accounted for by assuming it only functions in the P-L complex for mRNA synthesis. Curran (1996) demonstrated that addition of a wt P extract to an α-L immunoselected and concentrated P-L complex could restore transcriptional activity from 0% to 100%. He also showed that PΔ412-479, PΔ539-568, and PΔ446-568, which are P proteins containing deletions of the full L binding domain, the C-terminal NC binding domain, and the distal L binding and C-terminal NC binding domain, respectively, were all unable to rescue the wt polymerase. Thus, he concluded that rescue did not occur because the supplemental function requires that the free P somehow interact with both L associated in the bound polymerase complex and the template.

We also performed rescue experiments and obtained very fascinating results. These mutations may help to identify

the exact mechanism of the supplemental function of P protein since our mutations do not contain extensive deletions of the protein nor are they defective in any of the wild type protein-protein interactions necessary for mRNA synthesis. Our data showed that rescue by the addition of a wt P extract could be accomplished for the mutant polymerases K453A and L421A, but not for P408/9 or P4 (Figs. 18-20). Interestingly, the mutant P extracts could not fully rescue the wt polymerase although the L binding site is functional. These results indicate that mutants which are rescued may be defective in a supplemental role of P, while polymerase function remains intact. The mutants which could not be fully rescued and could not in turn rescue wild type polymerase may indicate a more severe compromise of function affecting both polymerase and supplemental activities. This is not an unreasonable assumption due to the apparent dual function of this domain. Mutations which interrupt a critical interaction necessary for proper conformation may lead to disruption of both activities.

We next wished to determine whether this excess P is rescuing activity by exchange between free P and that associated with the polymerase. There are several pieces of data which which suggest no exchange occurs. Sedimentation analyses (Fig. 21 and 22) support this hypothesis as well as the fact that two of the mutants could not be rescued. Previous data showed that the P and L proteins must be

coexpressed in order to form a complex as well as an active polymerase (Horikami et al., 1992). It has also been demonstrated that the P protein requires coexpression for oligomerization as determined by GST-P/P cobinding analyses (data not shown) and hemaglutinin epitope tagged P dilution assays (Curran et al., 1995). However, each of the protein assays used to determine exchange were done with the soluble proteins in the absence of the viral template. Thus, it cannot be ruled out that exchange occurs only during RNA synthesis while the polymerase and excess P protein are actively associated with the template.

Therefore, two models may be proposed as visualized in the cartoon in Fig. 29. The first considers that exchange does not occur and thus defines the supplemental role of the P protein to be independent of the polymerase complex, possibly being important for the removal of NP from the nucleocapsid such that the RNA is accessible to the viral polymerase. The second model would propose that exchange occurs between the P proteins of the polymerase and free P during RNA synthesis aiding in the movement of the polymerase down the template. Either of these models could have the same basic function: removal of the NP proteins from the RNA.

The RNA within the nucleocapsid is RNase insensitive at all times during viral RNA synthesis. Thus it is believed that the RNA must somehow be uncoated such that the L protein may be free to interact with the bases. It is logical to

A. THE INDEPENDENT MODEL

B. THE EXCHANGE MODEL

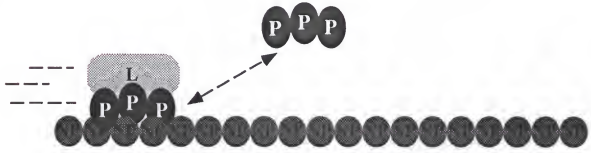


Fig. 29. Two hypothetical models for the mechanism of the supplemental function of the Sendai virus P protein.

assign this function to the P protein since it is responsible for binding the polymerase to the template. One could hypothesize that two NC binding domains are separated in such a way to allow the P protein, in essence, to stradle the template and possibly move the NP proteins from the template enough to expose the bases, but not enough to allow exposure of the RNA backbone to RNases. Under EM the nucleocapsid has been observed to have a relatively tight pitch of 5.3 or 6.8 nm and a more extended conformation with a pitch of 37.5 nm. Thus, P could be functioning in the transition between these pitch states which may be necessary to open the nucleocapsid helix for RNA synthesis. Future experiments should determine which model is correct, however, these studies have provided unique evidence for a functional role of P protein in mRNA synthesis and have stressed its central position in viral replication.

Preliminary data provides evidence that P408/9, P4, and K453A all synthesize leader (+) RNA at or near wild type levels. Thus, impairment of either polymerase or supplemental activity is not visualized upon synthesis of only the first 55 nt. The fact that the polymerases, even the most defective, are able to synthesize *le+* but not subsequent and longer RNAs, suggests the idea that P may have a role in the processivity of the enzyme. On the other hand, it also raises the idea that upon pausing at the intergenic regions, P is necessary for moving the polymerase into the

next transcriptional unit. Both of these questions could be explained by a hypothesis that P is important for removing or displacing the NP protein from the template to allow movement of the polymerase.

Of interest here, Gao and Lenard (1996) provided evidence that the VSV P-L complex is bound more tighly than P alone. If this is analogous for Sendai, then it would lend further evidence that P functions alone to increase the processivity of the polymerase, as it would be easier for the supplemental P to move on and off the template. Future experiments which determine the state of P oligomerization for supplemental function may prove extremely informative. If a P mutant which is unable to oligomerize can rescue transcription then it could be determined that oligomerization is unnecessary, or if it cannot rescue, then it is necessary. A requirement for oligomerization would be intriguing since many cellular transcription factors, such as Jun and Fos (Lewin, 1994) undergo coiled-coil multimerization as does SV P (Curran et al., 1995b).

Future directions first will be aimed at determining the effects of the P mutants in viral replication. Replication necessitates that synthesis of the viral RNA occurs concommitantly with encapsidation of progeny RNA by the NP-P complex. These studies should prove interesting, since upon initiation of encapsidation, the intergenic pause sites are presumably ignored. Thus, if P is necessary for moving the

polymerase after stalling at an intergenic region, it may be hypothesized to be irrelevent for this function during replication. Instead, the NP-P complex could act in a similar manner or the conformation of the polymerase could be such that a similar function is not mandatory. It should be noted that the binding sites for NC and NP_0 are discrete from the L binding site, and thus the interactions of the P proteins with NC or NP_0 should remain wild type.

These questions could initially be addressed by reconstitution assays in which the polymerase and encapsidation substrates are expressed separately and then mixed prior to *in vitro* replication analyses. This would provide a way to determine if either or both of the encapsidation or polymerase comlexes are impaired. Rescue studies could also be performed to determine if the supplemental role of P is required for replication as well as transcription. Each of these experiments should help to further guide our studies in determining the role of P in viral RNA synthesis.

In addition to the replication studies, further analysis of leader RNA synthesis must be performed for each of the defective P mutants. It may also be of interest to determine if the mutants are temperature sensitive with respect to transcription. This may provide further insight into the nature of the interactions. Finally, a very appealing

experiment would be to clone the mutant P proteins which rescue, K453A and L421A, into the full length infectious Sendai virus clone (Garcin et al., 1995) to test for their effect in the context of a virus infection. If any of the mutant viruses reverted back to wildtype, investigation of second site suppressors may prove to be very informative. In conclusion, this research has provided novel evidence for a supplemental role for the P protein in viral RNA synthesis and has opened many avenues for future investigations of the P protein.

REFERENCE

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.F., Smith, J.A., and Struhl, K., (ed.). (1987). Current Protocols in Molecular Biology, John Wiley & Sons, New York.

Baker, S.C., and Moyer, S.A. (1988). Encapsidation of Sendai virus genome RNAs by pufified NP protein during *in vitro* replication. J. Virol. 62:834-838.

Banerjee, A.K. and Barik, S. (1992). Gene expression of vesicular stomatiits virus genome RNA. Virology 188:417-428.

Bankamp, B., Horikami,S.M. Thompson,P.D., Huber, M., Billeter, M., and Moyer, S.A. (1996). Domains of the measles virus N protein required for binding to P protein and self-assembly. Virology 216:272-277.

Barik, S. and Banerjee,A.K. . (1991). Cloning and expression of the vesicular stomatitis virus phosphoprotein gene in E. coli: Analysis of phosphorylation status versus transcriptional activity. J. Virol. 65:1719-1726.

Barik, S. and Banerjee, A.K. (1992). Sequential phosphorylation of the phosphprotein of vesicular stomatitis virus by cellular and viral protein kinases is essential for transcriptional activation. J. Virol. 66:1109-1118.

Barik, S., McLean, T. and Dupuy, L.C. (1995). Phosphorylation of Ser232 directly regulated the transcriptional activity of the P protein of human respiratory syncytial virus: Phosphorylation of Ser237 may play an accessory role. Virology 213:405-412.

Bass, S.H., Mulkerrin, M.G., and Wells, J.A. (1991). A systematic mutational analysis of hormone-binding determinants in the human growth hormone receptor. Proc. Natl. Aca. Sci. USA 88:4498-4502.

Beckes, J.D. and Perrault, J.. (1992). Stepwise phosphorylation of vesicular stomatitis virus P protein by virion-associated kinases and uncoupling of

second step from in vitro transcription. Virology 188:606-617.

Beijerinck, M.W., van Iterson, G., den Dooren de Jong, L.E., and Kluyver, C.J. (1898). Ueber ein Contagium vivum fluidum als Ursache der Fleckenkrankheit der Tabaksblätter. Verh. K. Akad. Wet. 6:3-21. Translation: Johnson, J. (1942). Concerning contagium vivum fluidum as a cause of the spot-disease of tobacco leaves. Phytopath. Class. 7:33-52.

Bennett, W.F., Paoni, N.F., Keyt, B.A., Botstein, D., Jones, A.J.S., Presta, L., Wurm, F.M., and Zoller, M.J. (1991). High resolution analysis of functional determinants on human tissue-type plasminogen activator. J. Biol. Chem. 266:5191-5201.

Billeter, M., and Cattaneo, R. (1991). Molecular biology of defective measles viruses persisting in the human central nervous system. In The Paramyxoviruses, D. Kingsbury, Ed. Plenum, New York, pp. 323-345.

Black, F.L. (1991). Epidemiology of *Paramyxoviridae*. In The Paramyxoviruses, D. Kingsbury, Ed. Plenum, New York, pp.509-536.

Blumberg, B.M., Chan, J., and Udem, S.A.(1991). Function of paramyxovirus 3′ and 5′ end sequences in theory and practice. In The Paramyxoviruses, D. Kingsbury, Ed. Plenum Press, New York, pp. 235-247.

Blumberg, B.M., and Kolakofsky, D. (1981). Intracellular vesicular stomatitis virus leader RNAs are found in nucleocapsid structures. J. Virol. 40:568-576.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. 72:248-254.

Buchholz, C.J., Retzler, C., Homann, H.E., and Neubert, W.J. (1994). The carboxyl-terminal domain of Sendai virus nucleocapsid protein is involved in complex formation between phosphoprotein and nucleocapsid-like particles. Virology 204:770-776.

Buchholz, C.J., Spehner, D., Drillien, R., Neubert, W.J., and Homann, H.E. (1993). The conserved N-terminal region of Sendai virus nucleocapsid protein NP is required for nucleocapsid assembly. J. Virol. 67:5803-5812.

Byrappa, S., Hendricks, S., Pan, Y.-B., Seyer, J.M., and Gupta, K.G. (1995). Intracellular phosphorylation of the Sendai virus P protein. Virology 208:408-413.

Byrappa, S., Pan, Y.-B., and Gupta, K.C. (1996). Sendai virus P protein is constitutively phosphorylated at serine 249: High phosphorylation potential of the P protein. Virology 216:228-234.

Calain, P., Curran, J., Kolakofsky, D., and Roux, L. (1992). Molecular cloning of natural paramyxovirus copy-back defective interfering RNAs and their expression from DNA. Virology 191:62-71.

Calain, P., and Roux, L. (1993). The rule of six, a basic feature for efficient replication of Sendai virus defective interfering RNA. J. Virol. 67:4822-4830.

Canter, D.M., Jackson, R.L., and Perrault, J. (1993). Faithful and efficient *in vitro* reconstitution of vesicular stomatitis virus transcription using plasmid-encoded L and P proteins. Virology 194:518-529.

Carlsen, S.R., Peluso, R.W., and Moyer, S.A. (1985) *In vitro* replication of Sendai virus wild-type and defective interfering particle genome RNAs. J. Virol. 54:493-500.

Chandrika, R., Myers, T., and Moyer, S.A. (1995). Measles virus nucleocapsid protein can function in Sendai virus defective interfering particle genome synthesis in vitro. Virology 206:777-782.

Chattopadhyay, D. and Banerjee, A.K. (1987a). Phosphorylation within a specific domain of the phosphoprotein of vesicular stomatitis virus regulates transcription in vitro. Cell 49:407-414.

Chattopadhyay, D. and Banerjee, A.K. (1987b). Two separate domains within vesicular stomatitis virus phophoprotein support transcription when added in trans. Proc. Natl. Acad. Sci. USA 84:8932-8936.

Chattopadhyay, D. and Banerjee, A.K. (1988). NH2-terminal acidic region of the phosphoprotein of vesicular stomatitis virus can be functionally replaced by tubulin. Proc. Natl. Acad. Sci. USA 85:7977-7981.

Chinchar, V. G. and Portner, A. (1981). Functions of Sendai virus nucleocapsid polypeptides: Enzymatic activities in nucleocapsids following cleavage of polypeptide P by S. Aureus protease V8. Virology 109:59-71.

Collins, P.L. (1991). Molecular biology of RSV. In The Paramyxoviruses, D. Kingsbury, Ed. Plenum Press, New York, pp. 103-162.

Collins, P.L., Hill, M.G., Camargo, E., Grosfeld, H., Chanock, R.M., and B.R. Murphy, (1995). Production of infectious human respiratory syncytial virus from cloned cDNA confirms an essential role for the transcription elongation factor from the 5' proximal open reading frame of the M mRNA in gene expression and provides capability for vaccine development. Proc. Natl. Acad. Sci. USA 92:11563-11567.

Collins, P.L., Mink, M.A., Hill III, M.G., Camargo, E., Grosfeld, H., and Stec, D.S. (1993). Rescue of a 7502-nucleotide (49.3%) of full-length) synthetic analog of respiratory syncytial virus genomic RNA. Virology 195:252-256.

Collins, P.L., Mink, M.A., and Stec, D.S. (1991). Rescue of synthetic analogs of respiratory syncytial virus genomic RNA and effect of truncations and mutations on the expression of a foreign reporter gene. Proc. Natl. Acad. Sci. USA 88:9663-9667.

Conzelmann, K.K., and Schnell, M. (1994). Rescue of synthetic genomic RNA analogs of rabies virus by plasmid-encoded proteins. J. Virol. 68:713-719.

Crowley, J.C., Dowling, P.C., Mennona, J. Silverman, J.I., Shubak, D., Cook, S.D., and Blumberg, B.M. (1988). Sequence variability and function of measles virus 3'and 5' ends and intercistronic regions. Virology 164:498-506.

Cserhati, T., and Szogyi, M. (1995). Role of hydrophobic and hydrophilic forces in peptide-protein interaction: New advances. Peptides 16:165-173.

Curran, J., Boeck, R., and Kolakofsky, D. (1991a). The Sendai virus P gene expresses both an essential protein and an inhibitor of RNA synthesis by shuffling modules via mRNA editing. EMBO J. 10:3079-3085.

Curran, J., Boeck, R., Lin-Marq, N., Lupas, A., and Kolakofsky, D. (1995b). Paramyxovirus phosphoproteins form homotrimers as determined by an epitope dilution assay, via predicted coiled coils. Virology 214:139-149.

Curran, J., De Melo, M., Moyer, S., and Kolakofsky, D. (1991b). Characterization of the Sendai virus V protein with an anti-peptide antiserum. Virology 183:001-009.

Curran, J., Homann, H., Buchholz, C., Rochat, S., Neubert, W., and Kolakofsky, D. (1993). The hypervariable C-terminal tail of the Sendai paramyxovirus nucleocapsid protein is required for template function but not for RNA encapsidation. J. Virol. 67:4358-4364.

Curran, J., Marq, J.-B., and Kolakofsky, D. (1992). The Sendai virus nonstructural C proteins specifically inhibit viral mRNA synthesis. Virology 189:647-656.

Curran, J., Marq, J.-B., and Kolakofsky, D. (1995a). An N-terminal domain of the Sendai paramyxovirus P protein acts as a chaperone for the NP protein during the nascent chain assembly step of genome replication. J. Virol. 69:849-855.

Curran, J., Pelet, T., and Kolakofsky, D. (1994). An acidic activation-like domain of the Sendai virus P protein is required for RNA synthesis and encapsidation. Virology 202:875-884.

Cunningham, B.C., and Wells, J.A. (1989). High-resolution epitope mapping of hGH-receptor interactions by alanine-scanning mutagenesis. Science 244:1081-1085.

Das, T., Gupta, A.K., Sims, P.W., Gelfand, C.A., Jentoft, J.E., and Banerjee, A.K. (1995). Role of cellular casein kinase II in the function of the phosphoprotein (P) subunit of RNA polymerase of vesicular stomatitis virus. The Journal of Biological Chemistry 270:24100-24107.

De, B.P., and Banerjee, A.K. (1985). Requirements and functions of vesicular stomatitis virus L and NS proteins in the transcription process *in vitro*. Biochem. Biophys. Res. Commun. 126:40-49.

De, B.P., and Banerjee, A.K. (1993). Rescue of synthetic analogs of genome RNA of human parainfluenza virus type 3. Virology 196:344-348.

De, B.P., Gupta, S., and Banerjee, A.K. (1995). Cellular protein kinase C isoform ζ regulates human parainfluenza virus type 3 replication. Proc. Natl. Acad. Sci.92:5204-5208.

Deshpande, K.L., and Portner, A. (1984). Structural and functional analysis of Sendai virus nucleocapsid protein NP with monoclonal antibodies. Virology 139:32-42.

Deshpande, K.L. and Portner, A.(1985). Monoclonal antibodies to the P protein of Sendai virus define its structure and role in transcription. Virology. 140:125-134.

Diamond, S.E., and Kirkegaard, K. (1994). Clustered charged-to-alanine mutagenesis of poliovirus RNA-dependent RNA polymerase yields multiple temperature-sensitive mutants defective in RNA synthesis. J. Virol. 68:863-876.

Egelman, E.H., Wu, S.-S., Amrein, M., Portner, A., and Murti, G. (1989). The Sendai virus nucleocapsid exists in at least four different helical states. J. Virol. 63:2233-2243.

Einberger, H., Mertz, R., Hofschneider, P.H., and Neubert, W.J. (1990). Purification, renaturation, and reconstituted protein kinase activity of the Sendai virus Large (L) protein: L protein phosphorylates the NP and P proteins *in vitro*. J. Virol. 64:4274-4280.

Engelhorn, M., Stricker, R., and Roux, L. (1993) Molecular cloning and characterization of a Sendai virus internal deletion defective RNA. J. Gen. Virol. 74:137-141.

Fuerst, T.R., Niles, E.G., Studier, F.W., and Moss, B. (1986). Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. Proc. Natl. Acad. Sci. USA 83:8122-8126.

Fisher, L.E. (1990). Localization of antigenic sites in the primary sequence of the Sendai virus NP proein. Intervirology 31:116-121.

Fooks, A.R., Stephenson, J.R., Warnes, A., Dowsett, A.B., Rima, B.K., and Wilkinson, G.W.G. (1993). Measles virus nucleocapsid protein expressed in insect cells assembles into nucleocapsid-like structures. J. Gen. Virol. 74:1439-1444.

Galinski, M.S., and Wechsler, S.L. (1991). The molecular biology of the *Paramyxovirus* genus. In The Paramyxoviruses, D. Kingsbury, Ed. Plenum, New York, pp. 41-82.

Gao, Y., and Lenard, J. (1995a). Multimerization and transcriptional activation of the phosphoprotein (P) of vesicular stomatitis virus by casein kinase-II. EMBO J. 14:1240-1247.

Gao, Y. and Lenard, J. (1995b). Cooperative binding of the multimeric phosphoprotein (P) of vesicular stomatitis virus to polymerase (L) and template: Pathways of assembly. J. Virol. 69:7718-7723.

Garcin, D., Pelet, T., Calain, P., Roux, L., Curran, J., and Kolakofsky, D. (1995). A highly recombinogenic system for the recovery of infectious Sendai paramyxovirus from cDNA: generation of a novel copy-back nondefective interfering virus. EMBO J. 14:6087-6094.

Gill, D.S., Takai, S., Portner, A., and Kingsbury, D.W. (1988). Mapping of antigenic domains of Sendai virus nucleocapsid protein expressed in *Escherichia coli*. J. Virol. 62:4805-4808.

Goodheart, C.R. (1969). An Introduction to Virology. W.B. Saunders, Philadelphia, p. 1.

Gupta, K.C. and Kingsbury, D.W. (1984). Complete sequences of the intergenic and mRNA start signals in the Sendai virus genome: Homologies with the genome of the vesicular stomatitis virus. Nucleic Acids Research 12:3829-3841.

Hamaguchi, M., Yoshida, T., Nishikawa, K., Naruse, H., and Nagai, Y. (1983). Transcriptive complex of Newcastle disease virus. I. Both L and P proteins are required to constitute an active complex. Virology 128:105-117.

Hammond, O.C., Haley, B.E., and Lesnaw, J.A. (1992). Identification and characterization of serine/threonine protein kinase activity intrinsic to the L protein of vesicular stomatitis virus New Jersey. J. Gen. Virol. 73:67-75.

Hammond, O.C., and Lesnaw, J.A. (1987). Functional analysis of hypomethylation variants of the New Jersey serotype of vesicular stomatitis virus. Virology 160:330-335.

Hassett, D.E., and Condit, R.C. (1994). Targeted construction of temperature-sensitive mutations in vaccinia virus by replacing clustered charged residues with alanine. Proc. Natl. Acad. Sci. USA 91:4554-4558.

Heggeness, M.H., Scheid, A., and Choppin, P.W. (1980). Conformation of the helical nucleocapsids of paramyxoviruses and vesicular stomatitis virus: Reversible coiling and uncoiling induced by changes in salt concentration. Proc. Natl. Acad. Sci. USA 77:2631-2635.

Heggeness, M.H., Scheid, A., and Choppin, P.W. (1981). The relationship of conformational changes in the Sendai virus nucleocapsid to proteolytic cleavage of the NP polyeptide. Virology 114:555-562.

Hercyk, N., Horikami, S.M., and Moyer, S.A. (1988). The vesicular stomatitis virus L protein possesses the mRNA methyltransferase activities. Virology 163:222-225.

Higuchi, R., Krummel, B., and Saiki, R.K. (1988). A general method of *in vitro* preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions. Nucleic Acid Res. 16:7351-7367.

Horikami, S.M., Curran, J., Kolakofsky, D., and Moyer, S.A. (1992). Complexes of Sendai virus NP-P and P-L proteins are required for defective interfering particle genome replication *in vitro*. J. Virol. 66:4901-4908.

Horikami, S.M., and Moyer, S.A. (1991). Synthesis of leader RNA and editing of the P mRNA during transcription by purified measles virus. J. Virol. 65:5342-5347.

Horikami, S.M., and Moyer, S.A. (1995). Alternative amino acids at a single site in the Sendai virus L protein produce multiple defects in RNA synthesis in vitro. Virology 211:577-582.

Horikami, S.M., Smallwood, S., Bankamp. B., and Moyer, S.A. (1994). An amino-proximal domain of the L protein binds to the P protein in the measles virus RNA polymerase complex. Virology 205:540-545.

Hsu, C.-H., and Kingsbury, D.W. (1982). Topography of phosphate residues in Sendai virus proteins. Virology 120:225-234.

Huber, M., Cattaneo, R., Spielhofer, P., Örvell, C., Norrby, E., Messerli, M., Perriard, J.-C., and Billeter, M.A. (1991). Measles virus phosphoprotein retains the nucleocapsid protein in the cytoplasm. Virology 185:299-308.

Hughes, S.S. (1977). The Virus: A History of the Concept. Science History Publications, New York.

Hutchinson, K.L. (1993). Amino acid changes in the L polymerase proteins of vesicular stomatitis virus which confer aberrant polyadenylation and temperature-sensitive phenotypes. Virology 193:786-793.

Ivanovsky, D.I., and Poloftzoff, W. (1892). Über die Mosaikkrankheit der Tabakspflanze. Isv. imp. Akad. Nauk. 3:67-70. Translation: Johnson, J. (1942). Concerning the mosaic disease of the tabacco plant. Phytopath. Class. 7:27-30.

Jablonski, S.A., Luo, M., and Morrow, C. D. (1991). Enzymatic activity of poliovirus RNA polymerase mutants with single amino acid changes in the conserved YGDD amino acid motif. Virology 65:454565-4572.

Jin, H., and Elliot, R.M. (1992). Mutagenesis of the L protein encoded by Bunyamwera virus and production of monospecific antibodies. J. Gen. Virol. 73:2235-2244.

Kingsbury, D.W., (ed) (1991). The Paramyxoviruses. Plenum Press, New York.

Kolakofsky, D. (1976). Isolation and characterization of Sendai virus DI-RNAs. Cell 8:547-555.

Kolakofsky, D., Vidal, S., and Curran, J. (1991). Paramyxovirus RNA synthesis and P gene expression. In The Paramyxoviruses, D. Kingsbury, Ed. Plenum Press, New York, pp. 215-233.

Lamb, R. A., and Kolakofsky, D. (1996) Paramyxoviridae: The viruses and their replication. In Virology, B.N. Fields, Raven Press, New York. 1177-1204.

Lamb, R.A., Mahy, B.W.J., and Choppin, P.W. (1976). The synthesis of Sendai virus polypeptides in infected cells. Virology 69:116-131.

Lamb, R.A. and Paterson, R.G. (1991). The nonstructural proteins of paramyxoviruses. In The Paramyxoviruses, D. Kingsbury, Ed. Plenum Press, New York. 181-214.

Leppert, M., Kort, L., and Kolakofsky, D. (1977). Further characterization of Sendai virus DI-RNAs: a model for their generation. Cell 12:539-552.

Lewin, B. (1994). Genes V. Oxford University Press, New York.

Maniatis, T., Fritsch, E.F., and Sambrook,J. (1989). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Mayer, A. (1886). Ueber die Mosaikkrankheit des Tabaks. Landwn. Vers St-nen.32:451-467. Translation: Johnson, J.

(1942). Concerning the mosaic disease of tobacco. Phytopath. Class. 7:11-24.

Mazumder, B., Adhikary, G. and Barik, S.. (1994). Bacterial expression of human respiratory syncytial viral phosphoprotein P and identification of ser-237 as the site of phosphorylation by cellular casein kinase II. Virology. 205:93-103.

Mazumder, B. and Barik, S. (1994). Requirement of casein kinase II-mediated phosphorylation for the transcriptional activity of human respiratory syncytial viral phosphoprotein P: Transdominant negative phenotype of phosphorylation-defective P mutants. Virology 205:104-111.

Morgan, E.M. (1991). Evolutionary relationships of paramyxovirus nucleocapsid-associated proteins. In The Paramyxoviruses, D. Kingsbury, Ed. Plenum Press, New York, pp. 163-179.

Morgan, E.M., Re, G.G., and Kingsbury, D. (1984). Complete sequence of the Sendai virus NP gene from a cloned insert. Virology 135:279-287.

Moyer, S.A. (1989). Replication of the genome RNAs of defective interfering particles of vesicular stomatitis and Sendai viruses using heterologous viral proteins. Virology 172:341-345.

Moyer, S.A., Baker, S.C., Horikami, S.M. (1990). Host cell proteins required for measles virus reproduction. J. Gen. Virol. 71:775-783.

Moyer, S.A., Baker, S.C., and Lessard, J.L. (1986). Tubulin: a factor necessary for the synthesis of both Sendai virus and vesicular stomatitis virus RNAs. Proc. Natl. Acad. Sci. USA 83:5405-5409.

Moyer, S.A., and Horikami, S.M. (1991). The role of viral and host cell proteins in paramyxovirus transcription and replication. In The Paramyxoviruses, D. Kingsbury, Ed. Plenum Press, New York, pp. 249-274.

Moyer, S.A., Smallwood-Kentro, S., Haddad, A., and Prevec, L. (1991). Assembly and transcription of synthetic vesicular stomatitis virus nucleocapsids. J. Virol. 65:2170-2178.

Myers, T.A., and Moyer, S.A. (1997a). An amino-terminal domain of the Sendai virus nucleocapsid protein is

required for template function in viral RNA synthesis. (1997a). J. Virol. 71:918-924.

Myers, T.A., Peiters, A., Moyer, S.A. (1997b). A highly conserved region of the Sendai virus nucleocapsid protein contributes to the NP-NP binding domain. Virology 229:322-335.

Parker, J.C., and Richter, C.D. (1986). Viral diseases of the respiratory system. In The Mouse in Biomedical Research, H.L. Foster, J.D. Small, and J.C. Foxe, Eds. Academic Press, New York, pp. 109-158.

Parks, G.D. (1994). Mapping of a region of the paramyxovirus L protein required for the formation of a stable complex with the viral phosphoprotein P. J. Virol. 68:4862-4872.

Pattnaik, A.K., Ball, L.A., LeGrone, A.W., Wertz, G.W. (1992). Infectious defective interfering particles of VSV from transcripts of a cDNA clone. Cell 69:1011-1020.

Peeples, M.E. (1991). Paramyxovirus M proteins: Putting it all together and taking it on the road. In The Paramyxoviruses, ed. D. W. Kingsbury. Plenum Press, New York, 427-451.

Pelet, T., Curran, J., and Kolakofsky, D. (1991). The P gene of bovine parainfluenza virus 3 expresses all three reading frames from a single mRNA site. EMBO J. 10:443-448.

Pelet, T, Delenda, C, Gubbay, O, Garcin, D, and Kolakofsky, D. (1996). Partial characterization of a Sendai virus replication promoter and the rule of six. Virology 224:405-14.

Peluso, R.W., and Moyer, S.A. (1988). Viral proteins required for the *in vitro* replication of vesicular stomatitiis virus defective interfering particle genome RNA. Virology 162:369-376.

Perrault, J., Clinton, G.M., and McClure, M.A. (1983). RNP template of vesicular stomatitis virus regulates transcription and replication functions. Cell 35:175-185.

Poch, O., Blumberg, B.M., Bougueleret, L., and Tordo, N. (1990). Sequence comparison of five polymerases (L proteins) of unsegmented negative-strand RNA viruses: theoretical assignment of functional domains. J. Gen. Virol. 71:1153-1162.

Portner, A., and Murti, K.G. (1986). Localization of P, NP and M proteins on Sendai virus nucleocapsids using immunogold labeling. Virology 150:469-478.

Portner, A., Murti, K.G., Morgan, E.M., and Kingsbury, D.W. (1988). Antibodies against Sendai virus L protein: distribution of the protein in nucleocapsids revealed by immunoelectron microscopy. Virology 163:236-239.

Pringle, C.R. (1991). The genetics of paramyxoviruses. In The Paramyxoviruses, D. Kingsbury, Ed. Plenum Press, New York, pp. 1-39.

Radecke, F., Spielhofer, P., Schneider, H., Kaelin, K., Huber, M., Dotsch, C., Christiansen, G., and Billeter, M.A. (1995). Rescue of measles virus from cloned DNA. EMBO J. 44:5773-5784.

Re, G.G. (1991). Deletion mutants of paramyxoviruses. In The Paramyxoviruses, D. Kingsbury, Ed. Plenum Press, New York, pp. 275-298.

Ryan, K.W., and Kingsbury, D.W. (1988). Carboxyl-terminal region of Sendai virus P protein is required for binding to viral nucleocapsids. Virology 167:106-112.

Ryan, K.W., Morgan, E.M., and Portner, A. (1991) Two noncontiguous regions of Sendai virus P protein combine to form a single nucleocapsid binding domain. Virology 180:126-134.

Ryan, K.W., Murti, K.G., and Portner, A. (1990). Localization of P protein binding sites on the Sendai virus nucleocapsid. J. Gen. Virol. 71:997-1000.

Ryan, K.W., and Portner, A. (1990). Separate domains of Sendai virus P protein are required for binding to viral nucleocapsids. Virology 174:515-521.

Ryan, K.W., Portner, A., and Murti, K.G. (1993). Antibodies to paramyxovirus nucleoproteins define regions important for immunogenicity and nucleocapsid assembly. Virology 193:376-384.

Sanchez, A., Bishnu, P.D., and Banerjee, A.K. (1985). *In vitro* phosphorylation of NS protein by the L protein of vesicular stomatitis virus. J. Gen. Virol. 66:1025-1036.

Schnell, M.J., and Conzelmann, K.K. (1995). Polymerase activity of *in vitro* mutated rabies virus L protein. Virology 214:522-530.

Schnell, M.J., Mebatsion, T., and Conzelmann, K.K. (1994). Infectious rabies viruses from cloned cDNA. EMBO J. 13:4195-4203.

Sidhu, M.S., Menonna, J.P., Cook, S.D., Dowling, P.C., and Udem, S.A. (1993). Canine distemper virus L gene: Sequence and comparison with related viruses. Virology 193:50-65.

Sleat, D.E., and Banerjee, A.K. (1993). Transcriptional activity and mutational analysis of recombinant vesicular stomatitis virus RNA polymerase. Virology 67:1334-1339.

Smallwood, S., and Moyer, S.A. (1993). Promoter analysis of the vesicular stomatitis virus RNA polymerase. Virology 192:254-263.

Smallwood, S., Ryan, K.W., and Moyer, S.A. (1994). Deletion analysis defines a carboxyl-proximal region of Sendai virus P protein that binds to the polymerase L protein. Virology 202:154-163.

Stein, E.O., Fuerst, T.R., and Moss, B. (1989). Cap-independent translation of mRNA conferred by encephalomyocarditis virus 5′ sequence improves the performance of the vaccinia virus/bacteriophage T7 hybrid expression system. Proc. Natl. Acad. Sci. USA 86:6126-6130.

Tapparel, C. and Roux, L. (1996). The efficiency of Sendai virus genome replication: the importance of the RNA primary sequence independent of terminal complementarity. Virology 225:163-71.

Thomas, D., Newcomb, W.W., Brown, J.C., Wall, J.S., Hainfeld, J.F., Trus, B.L., and Stevens, A.C. (1985). Mass and molecular composition of vesicular stomatitis virus: A scanning transmission electron microscopy analysis. J. Virol. 54:598-607.

Tsai, C.-J., Shuo, L.L., Wolfson, H.J., and Nussinov, R. (1996). Protein-protein interfaces: Architectures and interactions in protein-protein interfaces and in protein cores. Their similarities and differences. Critical Reviews in Biochemistry and Molecular Biology 31:127-152.

Vidal, S., Curran, J., and Kolakofsky, D. (1990a). Editing of the Sendai virus P/C mRNA by G insertion occurs during mRNA synthesis via a virus-encoded activity. J. Virol. 64:239-246.

Vidal, S., Curran, J., and Kolakofsky, D. (1990b). A stuttering model for paramyxovirus P mRNA editing. EMBO. J. 9:2017-2022.

Vidal, S., Curran, J., Orvell, C. and Kolakofsky, D. (1988). Mapping of monoclonal antibodies to the Sendai virus P protein and the location of its phosphates. J. Virol. 62:2200-2203.

Vidal, S., and Kolakofsky, D. (1989). Modified model for the switch from Sendai virus transcription to replication. J. Virol. 63: 1951-1958.

Wertman, K.F., Drubin, D.G., and Botstein, D. (1992). Systematic mutational analysis of the yeast *ACT1* gene. Genetics 132:337-350.

Wertz, G.W., and Davis, N.L. (1979) RNase III cleaves vesicular stomatitis virus genome-length RNAs but fails to cleave viral mRNAs. J. Virol. 30:108-115.

Wertz, G.W., Whelan, S., LeGrone, A., and Ball, A. (1994). Extent of terminal complementarity modulates the balance between transcription and replication of vesicular stomatitis virus RNA. Proc. Natl. Acad. Sci. USA 91:8587-8591.

Whelan, S.P.J., Ball, L.A., Barr, J.N., and Wertz, G.T.W. (1995). Efficient recovery of infectious vesicular stomatitis virus entirely from cDNA clones. Proc. Nat. Acad. Sci. USA 92:8388-8392.

Zhao, H. and A. K. Banerjee. (1995). Interaction between the nucleocapsid protein and the phosphoprotein of human parainfluenza virus 3. J.Biol.Chem. 270:12485-12490.

BIOGRAPHICAL SKETCH

Mary Catherine Bowman was born in Winter Park, Florida, in 1969 to John Wallace and Donna Marie Bowman. She attended school in Winter Park where she graduated from Winter Park High School in 1987. She went on to attend the University of North Carolina at Chapel Hill and graduated there in 1990 with a degree in chemistry. She then matriculated into the University of Florida Medical College in 1991. After completing two years of the medical program, she switched into the M.D./Ph.D. program and became affiliated with the Department of Molecular Genetics and Microbiology. During her graduate career she was under the guidance of Dr. Sue A. Moyer. After receiving her Doctor of Philosophy, she will remain in Gainesville to complete her third and fourth years of medical school. After receiving her Doctor of Medicine, she plans on entering into a competitive residency program in either internal medicine or pathology. Eventually she would like to pursue a career in academic medicine and maintain an active involvement in research. She now lives with her 3-year-old Doberman pinscher, Jack, and her 2-year-old deaf Catahoula leopard dog, Sadie.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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